A NEW MAGNETIC RESONANCE IMAGING CONTRAST AGENT FOR THE DETECTION OF GLUTATHIONE

A Thesis
Presented to
The Academic Faculty

by

Amy Rebecca Guinn

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in the
School of Bioengineering

Georgia Institute of Technology
May 2006

A NEW MAGNETIC RESONANCE IMAGING CONTRAST AGENT FOR THE DETECTION OF GLUTATHIONE

Approved by:

Dr. Niren Murthy, Advisor
School of Biomedical Engineering
Georgia Institute of Technology

Dr. Xiaoping Hu
School of Biomedical Engineering
Georgia Institute of Technology

Dr. Dean P. Jones
Department of Medicine
Emory University

Date Approved: January 10, 2006

To my family

ACKNOWLEDGEMENTS

First and foremost I would like to thank my advisor, Dr. Niren Murthy, for all his guidance and patience. I would also like to thank all the members past and present of the Murthy lab including Steve Yang, Mike Heffernan, Scott Wilson, Bill Fox, and Dr. Seraj Kaja for all their help in my research efforts.

I would especially like to thank Mom, Dad, and John for all their support over these past few years. I would not be where I am today with out them. A special thanks to all my extended family members in the Atlanta area, it was nice to always have a friendly, smiling face when I needed one most.

To my roommates and friends, Audrey, Jillian, Matias, and Abby, thanks for making my time in grad school so much fun and being there when I needed a shoulder. I would also like to thank all my friends at Georgia Tech and in Atlanta for the good times and great intellectual conversations.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iv
LIST OF TABLES	vii
LIST OF FIGURES	viii
LIST OF SYMBOLS AND ABBREVIATIONS	ix
SUMMARY	xi
CHAPTER	
1 INTRODUCTION	1
Basic principles of magnetic resonance imaging	1
Agents that alter MR image contrast	4
Gd versus Dy	6
Macromolecular MRI contrast agents	10
Pegylated MRI contrast agents	11
A drawback to macromolecular MRI contrast agents	13
Importance of GSH	14
Implications of GSH imbalance	15
2 HYPOTHESIS AND SPECIFIC AIMS	16
Hypothesis 1	16
Specific Aim 1	16
Hypothesis 2	16
Specific Aim 2	17
3 MATERIALS AND METHODS	18
Materials	18
Synthesis of contrast agents 1 and 2	18

Synthesis of contrast agents 3 and 4 19
TNBS assay 22
T_2 measurements 23
T_2 pulse sequence 23
Synthesis of contrast agent 5 24
Synthesis of C 24
Synthesis of D 25
Synthesis of E 26
Synthesis of contrast agent 6 28
GSH experiments with 5 31
GSH experiments with 6 31
4 RESULTS 33
Reaction yields of contrast agents 1, 2, 3, and 4 33
T_2 measurements of contrast agents 1 and 3 34
T_2 measurements of contrast agents 2 and 4 38
Results of the synthesis of contrast agent 5 42
GSH T_2 experiments with contrast agent 5 43
Results of the synthesis of contrast agent 6 46
GSH T_2 experiments with contrast agent 6 46
5 DISCUSSION 49
6 CONCLUSIONS AND FUTURE WORK 56
Future Work 57
REFERENCES 58

LIST OF TABLES

	Page
Table 4-1: T_2 data for contrast agent 1	34
Table 4-2: T_2 data for contrast agent 3	35
Table 4-3: T_2 data for contrast agent 2	38
Table 4-4: T_2 data for contrast agent 4	39
Table 4-5: T_2 data for contrast agent 5 with GSH	44
Table 4-6: T_2 data for contrast agent 6 with GSH	47

LIST OF FIGURES

Page

Figure 1-1: Vector representing the net magnetization of the protons in the presence of an external magnetic field 1

Figure 1-2: 90º RF pulse (A), and longitudinal relaxation, T_1 (B) 3

Figure 1-3: Transverse relaxation, T_2 4

Figure 1-4: Rotational correlation time, water exchange rate, and coordinated water molecule for DTPA-Gd 6

Figure 1-5: Glutathione structure illustrating the three amino acids that comprise it (A) and the structure of the dimer, GSSG (B) 14

Figure 3-1: Synthesis of contrast agents 1 and 2 21

Figure 3-2: CPMGT2 signal pulse sequence 24

Figure 3-3: Synthesis of contrast agent 5 27

Figure 3-4: Synthesis contrast agent 6 30

Figure 4-1: Concentration (mM) versus 1/ T_{2obs} (s^{-1}) to calculate the R_2 value of 1 36

Figure 4-2: Concentration (mM) versus 1/ T_{2obs} (s^{-1}) to calculate the R_2 value of 3 37

Figure 4-3: Concentration (mM) versus 1/ T_{2obs} (s^{-1}) to calculate the R_2 value of 2 40

Figure 4-4: Concentration (mM) versus 1/ T_{2obs} (s^{-1}) to calculate the R_2 value of 4 41

Figure 4-5: NMR spectrum of product C 42

Figure 4-6: The average T_2 data for contrast agent 5 45

Figure 4-7: Percent change in T_2 for contrast agent 6 48

Figure 5-1: Expected route of de-pegylation of contrast agents 5 and 6 by GSH leading to a change in T_2 that can be detected by MRI 52

LIST OF SYMBOLS AND ABBREVIATIONS

MRI	magnetic resonance imaging
GSH	glutathione
DTPA	diethylenetriaminepentaacetic
Dy	dysprosium
PEG	poly(ethylene glycol)
kDa	kilo dalton
R_2	transverse relaxation rate
T_2	transverse relaxation
T_1	longitudinal relaxation
RF	radio frequency
Gd	gadolinium
R_1	longitudinal relaxation rate
FDA	Food and Drug Administration
MHz	mega hertz
DTPA-Dy	diethylenetriaminepentaacetic-dysprosium
DTPA-Gd	diethylenetriaminepentaacetic-gadolinium
mM	millimolar
s	second
T	Tesla
RBC	red blood cell
ns	nanosecond
GSSG	glutathione dimer
μM	micomolar
DMSO	dimethylsulfoxide

TEA	triethyl amine
DTT	dithiothreitol
Gly-Pro	glycine-proline
DTPA-BA	diethylenetriaminepentaacetic bisanhydride
$DyCl_3$	dysprosium chloride
$GdCl_3$	gadolinium chloride
PBS	sodium phosphate salt
MeOH	methanol
mg	milligram
mL	milliliter
µL	microliter
TNBS	trinitrobenzene sulfonic acid
M	molar
w/v	weight per volume
NMR	nuclear magnetic resonance
GSH	glutathione
cm	centimeter
UV	ultra violet
UV-Vis	ultra violet-visible

SUMMARY

Magnetic Resonance Imaging (MRI) is an ideal candidate for diagnostic development and drug efficacy studies due to its non-invasive character, deep tissue imaging capabilities, and clinical availability. Currently, there is much interest in developing new MRI contrast agents to enhance its imaging ability and detect biological events *in vivo*. Improving MRI contrast agents could lead to earlier diagnosis of pathologies and better determination of drug activity.

This work concentrates on engineering a new MRI contrast agent that can detect the biologically important molecule, glutathione (GSH). A GSH-sensitive MRI contrast agent was designed and synthesized, which consists of a dysprosium-DTPA (DTPA-Dy) ligand conjugated to a 20kDa molecular weight poly(ethylene glycol) (PEG) chain through a disulfide bond. This contrast agent, in the presence of GSH, is expected to undergo a disulfide exchange reaction, which will remove the PEG chain from the DTPA-Dy resulting in a decrease in its transverse relaxation rate (R_2), and an increase in T_2.

This design is based on preliminary results presented here, which demonstrate that adding a PEG chain to DTPA-Dy increases its contrast ability (R_2 is increased). A PEG chain with a molecular weight of 5kDa increases the R_2 of DTPA-Dy from approximately $1mM^{-1}s^{-1}$ to about $6mM^{-1}s^{-1}$. If a larger PEG chain (20kDa) is added the R_2 of DTPA-Dy is further increased to about $12mM^{-1}s^{-1}$.

To test the design of the GSH-sensitive MRI contrast agent, several samples were made in buffer solution and their T_2 values were measured before and at two time points after

the addition of GSH. The control for this experiment was a sample of the contrast agent in buffer that was measured at the same time points as the experimental groups without the addition of GSH. The addition of GSH to solutions containing the contrast agent produced a 72% change in T_2 over a 22% change in the control.

The results of this work demonstrate that the R_2 of a Dy-based MRI contrast agent can be manipulated by altering the length of the PEG chain conjugated to DTPA-Dy. This new strategy has the potential to be used to design other biologically sensitive MRI contrast agents to detect a wide variety of biomolecules.

CHAPTER 1

INTRODUCTION

Basic principles of magnetic resonance imaging

Magnetic resonance imaging (MRI) is based on applying an external magnetic field to water protons. When the external magnetic field is applied to these protons, which are oriented in random directions, they become aligned with the external magnetic field. The protons can align either with the magnetic field (referred to as the positive z direction, +z) or opposite to the field (referred to as the negative z direction, -z). These oriented spins remain constant and form the net magnetization (Figure 1-1) [1].

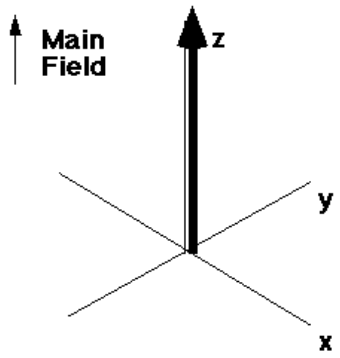

Figure 1-1 Vector representing the net magnetization of the protons in the presence of an external magnetic field [2]

To collect an MRI signal, a radio frequency (RF) pulse (Figure 1-2) is applied to the aligned protons that tips their axis of rotation from the +z direction into the x-y plane. This is called the 90º pulse. When this pulse is removed from the system, the protons then emit energy to re-align with the z axis. It is this emitted energy that the MRI machine measures and it decays with time as more and more protons re-align along the z axis.

Relaxation is the process by which protons emit the energy that was absorbed from the RF pulse and is the primary mechanism for image contrast. In a MRI scan, two relaxation times are gathered, T_1 and T_2, these variables are the measure of the energy emitted by an excited proton.

The longitudinal relaxation time, T_1, is the measure of the time required for 63% of the protons to re-align along the z axis after the 90º pulse (Figure 1-2). T_1 is also sometimes referred to as the spin-lattice relaxation. Immediately after the 90º RF pulse, the protons have no longitudinal magnetization (they are all rotated into the x-y plane), as time elapses, an exponential increase in longitudinal magnetization will be observed as the protons release their absorbed energy. For T_1 relaxation, it is vital for the protons to release their energy to their surroundings. If there is another entity present in the environment of the proton that can absorb this energy, such as a metal, protein, etc., then the bulk T_1 is altered. T_1 is shortened when this energy transfer is efficient.

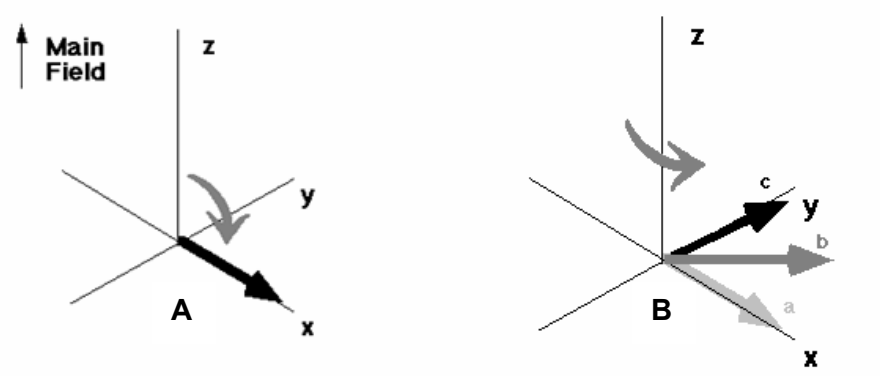

Figure 1-2 90º RF pulse (A), and longitudinal relaxation, T_1 (B) [2]

The transverse relaxation time (or spin-spin relaxation), T_2, is the measure of the time required for the transverse component of the net magnetization to decay to 37% of its original value (Figure 1-3). When the protons are rotated into the x-y plane by the 90º RF pulse, they lose coherence as they re-gain the z direction. The value of the net magnetization decreases towards zero as the protons' coherence disappears. This decay occurs through an irreversible process when protons release their energy to other nearby protons. Small variations in a proton's microenvironment will cause energy loss. To remove any coherence loss due to external field inhomogeneity, a second RF pulse is applied at a time, τ, after the initial 90º RF pulse. This is a 180º RF pulse that rotates the protons into the negative x-y plane removing any external effects and allows for only measuring of the true T_2. The 180º RF pulses are continued until the protons completely de-phase [1].

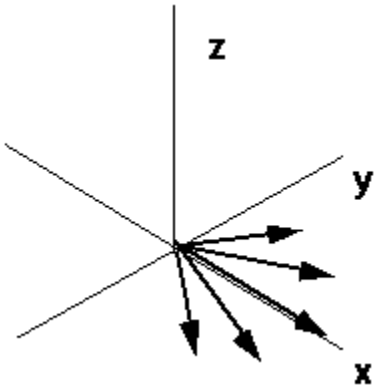

Figure 1-3 Transverse relaxation, T_2 [2]

The overall MR image is created by combining the T_1 and T_2 data in the following equation:

$$M(T_E) = M_0[1-e^{(-T_R/T_1)}]e^{(-T_E/T_2)}$$

In this equation M_0 is the total net magnetization, T_R(s) and T_E(s) are values input by the machine user, and T_1 and T_2 are the relaxation times of the solution measured [3].

Agents that alter MR image contrast

The main goal of MRI contrast agents is to improve MR images by adding functional data to the morphological information gathered from unenhanced images. MR images are created from proton relaxation times (T_1 and T_2) and from proton concentrations. The presence of a biological marker of interest can be imaged if it alters any of these two variables. Most contrast agents are chosen for their ability to alter T_1 or T_2 of the protons in their local environment. These protons will experience a varying magnetic field that depends on the magnetic moment of the contrast agent, its electronic spin relaxation

time, and the nature of the contrast agent's molecular interactions with the protons. These molecular interactions include inner-sphere and outer-sphere coordination and diffusional translation. T_1 and T_2 can be affected by the various properties of the contrast agents, but usually not to the same extent.

There are two major groups of MRI contrast agents. One consists of paramagnetic positive enhancers that increase the MRI signal intensity and the other consists of contrast agents that decrease MRI signal intensity by increasing the magnetic field inhomogeneity. Positive enhancers include manganese (II), iron (III), Gd (III), and stable free radicals such as nitroxides. These contrast agents have several unpaired electrons that cause them to be highly paramagnetic. They can be about 1800 times stronger than the hydrogen proton, which has only one unpaired electron. Negative MRI signal enhancers include superparamagnetic particles such as iron oxide particles and metal ions such as Dy (III). These particles alter proton relaxation times by creating microenvironments of magnetic field inhomogeneity. Protons located in or near these microenvironments will experience this field inhomogeneity, called the outer-sphere effect or susceptibility effect, and their spins will rapidly dephase resulting in a decrease in T_2. These effects are best seen using T_2-weighted and gradient-echo pulse sequences [4].

There are several characteristics of MRI contrast agents that can enhance their ability to alter proton relaxation times. As described above, contrast agents will usually enhance either T_1 or T_2 more significantly than the other. Therefore augmenting following characteristics will usually make the contrast agent a better T_1 or T_2 contrast agent. The important characteristics (Figure1-4) include water exchange rate, which is the rate of the coordinated water molecule exchanging with the bulk (also called inner-sphere

effects), rotational correlation time, which is how quickly the contrast agent is tumbling in solution, and susceptibility effect, which is the effect of the contrast agent on the bulk water (non-coordinated water molecules, also called outer-sphere effect).

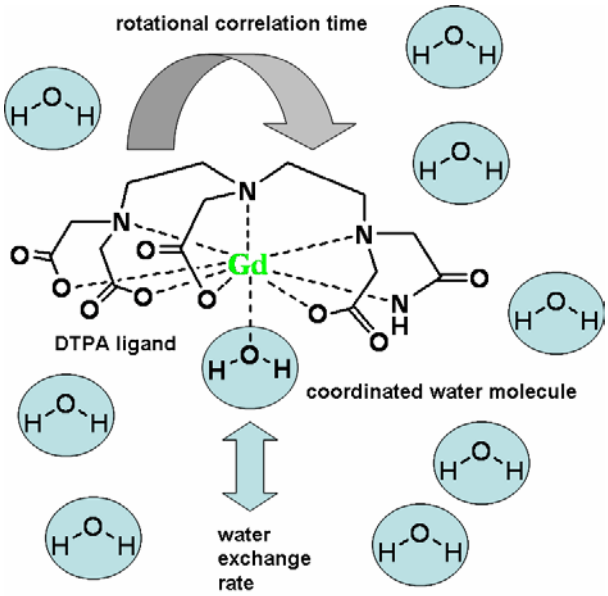

Figure 1-4 Rotational correlation time, water exchange rate, and coordinated water molecule for DTPA-Gd

Gd versus Dy

In this work, two contrast agents will be studied, the first and most well-known is a positive enhancer, Gd, and the other is a negative enhancer, Dy. Gd is currently the only MRI contrast agent approved for human use and is therefore more thoroughly studied in the literature and will be used as the basis for understanding Dy.

Gd is a positive T_1 enhancer, even thought it can also alter T_2, it is much more efficient at changing the longitudinal relaxivity of water protons. The current FDA approved Gd contrast agents are designed from DTPA-Gd and only possess a small percentage of the theoretical maximum longitudinal relaxivity (R_1). In a 20MHz field, current contrast agents only exhibit a longitudinal relaxivity of approximately $5mM^{-1}s^{-1}$. To design a Gd-based contrast agent with optimal R_1 properties, the molecular tumbling should be slowed (increase rotational correlation time) with more inner-sphere water molecules coordinated on the metal ion that have relatively short residence times. All three of these properties work "hand-in-hand." Usually, by optimizing one of these variables a decrease in one of the other variables off-sets the gain in R_1 and the overall contrast ability changes only slightly. The outer-sphere or susceptibility effect is very small for Gd-based contrast agents and is usually neglected. An example of the challenges faced when designing a Gd-based contrast agent is the attempt by researchers to increase their rotational correlation time grafting macromolecules to DTPA-Gd. Any increase in rotational correlation time is usually off-set by a decrease in the water exchange rate, which is undesirable for Gd, and therefore the overall effect is a small to modest increase in R_1 [5].

There have also been several attempts to increase the number of inner-sphere coordinated water molecules and shorten their residence times. These contrast agents have been more successful at increasing R_1 than the macromolecular Gd contrast agents. They have been able to increase the number coordinated water molecules from one in DTPA-Gd to two, as well as speed up the exchange rate of these water molecules, effectively increasing R_1 [6].

There is increasing interest in Dy-based contrast agents, since MRI technology is moving towards higher field strengths (>4T) and is rapidly reaching the limit where Gd-based contrast agents have poor water relaxivity [7]. Dy is a negative enhancer and has the most effect on the transverse relaxation (T_2) of the surrounding protons, so the design of an optimal Dy-based MRI contrast agent follows a different set of rules than Gd-based contrast agents. By increasing the compartmentalization of the Dy-based contrast agent, a large increase in transverse relaxivity of the bulk protons is achieved. This susceptibility or outer-sphere effect is caused by Dy creating regions of magnetic field inhomogeneity through which the water molecules diffuse. This effect is much more pronounced in Dy-based contrast agents than in Gd-based contrast agents. There have been several experiments to illustrate the susceptibility effect of Dy on the bulk transverse relaxivity. Fossheim et. al. injected red blood cells (RBCs) with DTPA-Dy and measured the R_2 of the solution. They compared this data to the same concentration of DTPA-Dy in solution and found a significantly larger R_2 for the solution with the RBCs containing DTPA-Dy. Hemolysis of the RBC suspension removed the compartmentalization effect and the R_2 decreased to about the same as the homogenous DTPA-Dy solution [8]. Wang et. al. used DTPA-Dy to visualize the difference between intact tumor tissue, where the DTPA-Dy would be compartmentalized around the cells, and areas of necrosis, where DTPA-Dy would be distributed homogenously. The MRI signal around the intact cells had a much lower T_2 signal than the areas of necrosis, allowing the researchers to definitively determine extent of necrosis in a colon cancer animal model [9].

In contrast to Gd-based contrast agents, the optimal condition for Dy-based contrast agents is a longer residence time of the coordinated water molecule. The transverse relaxivity of Dy-based contrast agents increases with the square of the external magnetic

field and the residence time of the coordinated water molecule [7, 10]. Therefore at low magnetic field strengths, water residence time has little effect, but increases with increasing field strength. For example, Elst et. al. found that at low field strengths, DTPA-Dy has an R_2 of less than one, but at higher field strengths it increases to about $1.5 mM^{-1}s^{-1}$ at 11.75T. Even though DTPA is not an optimal ligand, due to its fast water exchange rate (29ns), it exhibits this trend [7].

To slow the water exchange rate of the coordinated water molecule, amide bonds have been added to the DTPA ligand in a position where the amide oxygen would be one of the nine coordinated species on the metal ion. The amide oxygen is a weaker electron donor to the metal ion, which, in the ligand structure with the metal ion, would reduce crowding and allow the water molecule to remain bonded longer [11]. In Gd-based systems, ligands are designed to increase crowding so that the water exchange rate is subsequently increased [6]. The addition of amide bonds causes a decrease in the water exchange rate for the contrast agent in comparison to DTPA-Dy; it is hypothesized that one amide bond would cause a 3-4 fold decrease and two amide bonds would cause a 10 fold decrease in water exchange rate [11]. Elst et. al. demonstrated that at the same temperature and magnetic field strength, a Dy ligand with two amide bonds has a water exchange rate of 220-225ns while DTPA-Dy (zero amide bonds) has a water exchange rate of 29ns [7]. The ligand with the two amide bonds is a more efficient Dy contrast agent and has a higher R_2 value than DTPA-Dy. A word of caution, Elst et. al. found that if the water exchange rate is slowed too much, there is a deleterious effect on R_2 [10].

One other variable that is important in designing an efficient Dy-based contrast agent is the number of coordinated water molecules. Caravan et. al. reports that Dy-based

contrast agents need at least one coordinated water molecule to exhibit the quadratic increase in transverse relaxivity with magnetic field strength. They compared two Dy ligands, the first had one coordinated water molecule and the other had zero coordinated water molecules. The ligand that lacked the coordinated water exhibited no increase in R_2 with increasing field strength [12].

Macromolecular MRI contrast agents

Since DTPA-Gd and other small molecule Gd-based contrast agents have low retention in the blood (called "blood pool retention time") due to the fast clearance by the kidneys and liver, many researchers have attempted to increase this retention time by grafting macromolecules to the Gd ligand. Longer blood pool retention times are important for some imaging scans, especially for the cardiovascular system and tumor imaging. The researchers hoped that by adding a macromolecule to the ligand, there would be an increase the longitudinal relaxivity of the contrast agent by increasing its rotational correlation time. For most biocompatible linear polymer chains grafted to DTPA-Gd the increase in R_1 was far less than expected due to the high flexibility of the chains [11]. Some of the macromolecules that were successful in increasing the rotational correlation time of DTPA-Gd were dendrimers [13, 14], protein-ligand conjugates [15], and liposomes [16]. An example of liposomes that were able to increase R_1 of DTPA-Gd were made of egg lecithin, cholesterol, DTPA-Gd, and PEG and had a particle size of 205nm. These liposomes had and R_1 of about $17 mM^{-1}s^{-1}$ at a magnetic field strength of 10.7MHz, significantly higher than DTPA-Gd (the R1 of DTPA-Gd at 20MHz is $3.7 mM^{-1}s^{-1}$) [16]. A dendrimer that was successful at increasing the rotational correlation time of DTPA-Gd was designed by Langereis et. al. and had a R_1 of 19.7 $mM^{-1}s^{-1}$ (dendrimer

had a molecular weight of 65kDa), which is almost 5 times the R_1 of DTPA-Gd (4.2 $mM^{-1}s^{-1}$) at 1.5T. They found a linear relationship between the number of dendrimer generations (higher the number of generations, the larger the molecular weight of the macromolecule) to the R_1 value measured [13].

Pegylated MRI contrast agents

One of the highly biocompatible, linear polymers that is well studied and exhibits unique properties when used for contrast agents is PEG. Incorporating PEG either physically or covalently to the Gd-based contrast agent will increase the blood pool retention time and prevent unwanted liver accumulation of the contrast agent [17]. It is also known that for PEG, the optimal molecular weight for blood pool retention is 20kDa or greater [18].

PEG is a FDA approved polymer that can be injected, applied topically and rectally, and inhaled into the nasal cavity. It has been used extensively for drug delivery applications to decrease clearance of the agent and protect it from serum proteins. Chemically attaching a PEG moiety to an agent is called pegylation. Pegylation changes, in a desirable fashion, the immunological, pharmacokinetic, and pharmacodynamic properties of the agent to which it is bonded. Some of these advantages are increased water solubility, decreased renal clearance, and reduced toxicity. Pegylation also increases the molecular weight of the agent with the effective molecular weight greater than the apparent molecular weight, since PEG is heavily hydrated and in constant, rapid kinetic motion. This increase in molecular weight creates a protective barrier around the agent, consisting of the polymer and its associated water molecules, that protects the agent from immunogenic recognition and degradation by proteolytic enzymes [19, 20]. PEG strongly hydrogen bonds to the surrounding water molecules

and competes with water-water hydrogen bonding, creating a large hydrodynamic radius [21, 22, 23]. PEG can also direct the elimination route of the agent to which it is attached; for instance, PEG of molecular weight 6kDa four hours post injection is 92% in the excrement, 0.5% in the blood, 2.5% in the liver and 0.3% in the kidneys, while an injection of PEG of 20kDa is 42% in the excrement, 19% in the blood, 2.5% in the liver, and 0.5% in the kidneys [24]. The elimination rate of PEG moieties can be altered by administering via different routes. Intraperitoneal has the fastest elimination rate followed by subcutaneous then intramuscular injection [25].

Since the addition of PEG to MRI contrast agents provides it with desirable effects such as longer blood pool retention times and excretion routes, its effects on the metal ion core have been further studied. Although the molecular weight of the contrast agent is increased significantly, very little increase in relaxivity is found [18, 26]. PEG has very little effect on the rotation correlation times of the contrast agents to which it is conjugated, since it contributes almost no additional rigidity to the structure [11, 27]. If PEG is conjugated to a more rigid structure, such as a dendrimer, it will actually reduce the T_1 relaxivity, so any increase in R_1 from an increase in rotational correlation time is off-set by some decrease in another variable by PEG [17]. Doble et. al. synthesized a new Gd ligand that allows two water molecules to be coordinated on the Gd ion which increased R_1 significantly. When the researchers grafted PEG chains of various molecular weights to the ligand, the number of coordinated water molecules decreased from two to one, reducing the R_1 close to that of DTPA-Gd [27]. This is hypothesized to be the trade-off when adding PEG to a contrast agent, any minor increase in rotational correlation time is off-set by the undesirable effect (for Gd) of longer water residence times. The estimated increase in water residence time is a factor of four for the addition of a PEG chain with a molecular weight of 5kDa [5].

All the undesirable effects of grafting PEG to a contrast agent, a reduction in coordinated water molecules and a reduction in the water exchange rate, are only undesirable for Gd-based contrast agents. Since Dy-based contrast agents are governed by a different set of rules, it seems possible that grafting PEG to Dy ligands would actually increase its transverse relaxivity. Dy-based contrast agents would benefit from an increase in rotational correlation time, fewer coordinated water molecules, and a reduction in water exchange rate. One of the goals of this work is to test this hypothesis to see if Dy-based contrast agents do benefit from the addition of PEG to the metal ligand.

A drawback to macromolecular MRI contrast agents

One of the drawbacks of grafting macromolecules to a contrast agent is that it is difficult for the contrast agent to be eventually (after the scan time) broken down and excreted by the body. Researchers have attempted to attach biodegradable polymers to the ligand, but this does not necessarily correct this problem, because the contrast agent must still be broken down in the lysosome and will therefore come in contact with the cell [28]. The lysosome has an acidic environment (pH 5.0) that can cause the dissociation of toxic Gd^{3+} from its ligand. Studies have shown that some of the toxic Gd^{3+} is not excreted and incorporated into bone [29]. Two designs were employed to utilize the extra cellular concentration of free thiols to degrade the contrast agent over time and avoid necessity of cellular uptake. One design attached the metal ligand to the polymer backbone through a disulfide and the other incorporated the disulfide into the polymer backbone. Both of these designs achieved a longer blood pool retention time with eventual clearance by the kidneys [30, 31, 32].

Importance of GSH

Living organisms maintain tight control of the extracellular redox state. One mechanism by which cells and tissues maintain a specific redox state is through the thiol/disulfide reaction of GSH. GSH is a simple molecule comprised of three amino acids, one of which is a cysteine that contains a free thiol. The reduced state of GSH is the monomer form with the free thiol present, while the oxidized form of GSH is the dimer, or disulfide reaction between two GSH molecules (denoted GSSG). In humans, one third of all free thiols present in the body are in the extracellular space such as blood plasma and lining fluids like mucus [34]. The majority of extracellular GSH is acquired from food sources and is absorbed through in the lumen of the small intestine [33]. Variations in the extracellular redox state can alter a cell's proliferation, differentiation, and apoptosis pathways significantly [34].

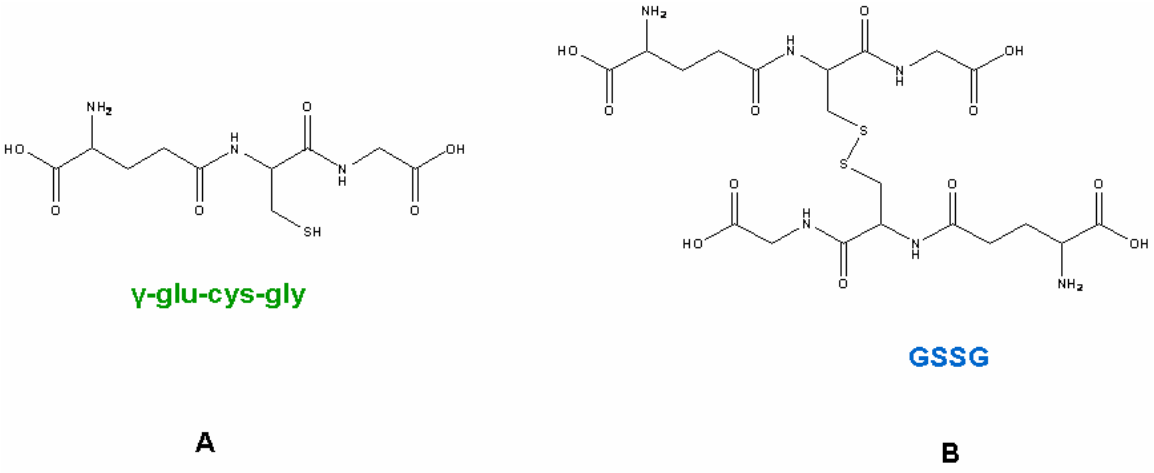

Figure 1-5 Glutathione structure illustrating the three amino acids that comprise it (A) and the structure of the dimer, GSSG (B)

The majority of extracellular GSH is in the reduced form with the free thiol present. In the plasma there is 2-4μM of GSH, in the lumen of the intestine there is milimolar concentrations and in the lining of the lungs the concentration of GSH is greater than 400 μM. Interestingly, in the central nervous system GSH is present in the oxidized, or dimer, form and any increase in GSH that leads to a more reduced state is deleterious to the surrounding cells [34].

Implications of GSH imbalance

Any imbalance between oxidants and anti-oxidants produce a condition called oxidative stress that contributes to most age-related diseases. These diseases include atherosclerosis, chronic lung disease, Alzheimer's disease, and Parkinson's disease [34]. A study by Sechi et. al. demonstrated that patients with severe Parkinson's disease have a decreased amount of GSH in the nigra area of the brain and in patients with highly advanced Parkinson's disease GSH was virtually absent from the nigra [35]. With aging, all extracellular environments become more oxidized from decreased GSH levels and lower levels of GSH have been implicated in oxidative stress diseases. Treating patients with GSH may minimize the damage to tissues from oxidative stress by removing the problematic free radicals [34].

CHAPTER 2

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis 1

If the molecular weight of the Dy is increased through pegylation, then its T_2 relaxivity will increase, due to a slower water exchange rate, a greater susceptibility effect and an increase in rotational correlation time.

Specific Aim 1

Synthesize Dy-based contrast agents with different molecular weight PEGs and measure their T_2 values. Follow the same process to make different molecular weight Gd contrast agents for comparison.

Hypothesis 2

If the molecular weight of the Dy is changed from high to low by de-pegylation through the disulfide exchange reaction with glutathione, then glutathione concentrations can be detected by MRI.

Specific Aim 2

Synthesize a contrast agent with 20kDa PEG linked to DTPA-Dy through a disulfide. Measure the cleavage of the disulfide linkage in the presence of glutathione through a change in T_2.

CHAPTER 3

MATERIALS AND METHODS

Materials

All materials were used as received unless otherwise noted. The following materials were purchased from Nektar: mPEG-NH_2-5kDa, mPEG-NH_2-20kDa, mPEG-SH-20kDa, mPEG-OPSS-20kDa. The following materials were purchased from Sigma-Aldrich: dimethylsulfoxide (DMSO, dried over 4Å molecular sieves), triethyl amine (TEA), diethylenetriaminepentaacetic bisanhydride (DTPA-BA), aldrithiol, acetic acid, cysteamine, silica gel, dithiothreitol (DTT), glutathione (GSH), trinitrobenzene sulfonic acid (TNBS) and glycine-proline (Gly-Pro). The final materials were purchase from various vendors; dysprosium chloride ($DyCl_3$) and gadolinium chloride ($GdCl_3$) were purchased form Alfa Aesar, sodium phosphate salt (PBS) and sodium bicarbonate salt were purchased from J.T. Baker, methanol (MeOH) and ethyl acetate were purchased from EMD, and finally the PD-10 columns and dialysis bags were purchased from Pierce.

Synthesis of contrast agents 1 and 2

For the synthesis of contrast agents 1 and 2 (Figure 3-1), 513mg (0.1mmol) of mPEG-NH_2-5kDa was dissolved in 1mL of DMSO by vortexing the mixture in a 5mL round bottom flask equipped with a magnetic stir bar. 100µL (0.72mmol) of TEA was added to the flask and stirred over a magnetic stir plate for one minute. After that, 144mg (0.4mmol) of DTPA-BA was added to the flask and was dissolved by stirring. The

mixture was sealed with a rubber septum and allowed to react for 24 hours at room temperature. The reaction products were then purified by a PD-10 column, using deionized water as the elution solvent, to remove any unreacted DTPA-BA. The product fraction (A) was collected from the PD-10 column and freeze-dried for 24 hours (collected 489.2mg, or 95% of original mPEG-NH_2-5kDa amount). To complete the synthesis of 1, 63.5mg (0.012mmol) of A was dissolved in 2mL of deionized water in a scintillation vial and then 50mg (0.18mmol) of $DyCl_3$ was added to the solution. The mixture was stirred with a magnetic stir bar for 4 hours at room temperature after which it was purified by a PD-10 column to remove any unreacted $DyCl_3$. The product fraction was collected and freeze-dried for 24 hours. 63mg (99% of original A amount) of a white, fluffy solid was collected. To complete the synthesis of 2, 50mg (0.009mmol) of A was dissolved in 2mL of deionized water in a scintillation vial and then 50mg (0.19mmol) of $GdCl_3$ was added to the solution. The mixture was stirred with a magnetic stir bar for 4 hours at room temperature after which it was purified by a PD-10 column to remove any unreacted $GdCl_3$. The product fraction was collected and freeze-dried for 24 hours. 41mg (82% of original A amount) of a white, fluffy solid was collected. To determine reaction yields, a TNBS assay was performed on mPEG-NH_2-5kDa and product A.

Synthesis of contrast agents 3 and 4

For the synthesis of contrast agents 3 and 4 (Figure 3-1), 507mg (0.025mmol) of mPEG-NH_2-20kDa was dissolved in 1mL of DMSO by vortexing the mixture in a 5mL round bottom flask equipped with a magnetic stir bar. 28µL (0.02mmol) of TEA was added to the flask and stirred over a magnetic stir plate for one minute. After that, 36mg (0.1mmol) of DTPA-BA was added to the flask and was dissolved by stirring. The

mixture was sealed with a rubber septum and allowed to react for 24 hours at room temperature. The reaction products were then diluted by the addition of 8mL of deionized water and separated into three dialysis bags for purification (3,500 molecular weight cut-off). Deionized water was used as the dialysis solvent, and changed 4 times (total water used was 10L), to remove any unreacted DTPA-BA and DMSO. The product solution was collected from the dialysis bags and freeze-dried for 24 hours (collected 221.8mg, or 44% of original mPEG-NH_2-20kDa amount; one of the dialysis bags burst). To complete the synthesis of 3, 55.4mg (2.7μmol) of B was dissolved in 2mL of deionized water in a scintillation vial and then 50mg (0.18mmol) of $DyCl_3$ was added to the solution. The mixture was stirred with a magnetic stir bar for 4 hours at room temperature after which it was purified by dialysis against deionized water (3,500 molecular weight cut-off; 4 water changes for a total of 10L) to remove any unreacted $DyCl_3$. The product solution was collected and freeze-dried for 24 hours. 52mg (94% of original B amount) of a white, fluffy solid was collected. To complete the synthesis of 4, 55mg (2.7μmol) of B was dissolved in 2mL of deionized water in a scintillation vial and then 50mg (0.19mmol) of $GdCl_3$ was added to the solution. The mixture was stirred with a magnetic stir bar for 4 hours at room temperature after which it was purified by dialysis (3,500 molecular weight cut-off; 4 water changes for a total of 10L) to remove any unreacted $GdCl_3$. The product solution was collected and freeze-dried for 24 hours. 43mg (78% of original B amount) of a white, fluffy solid was collected. To determine reaction yields, a TNBS assay was performed on mPEG-NH_2-20kDa and product B.

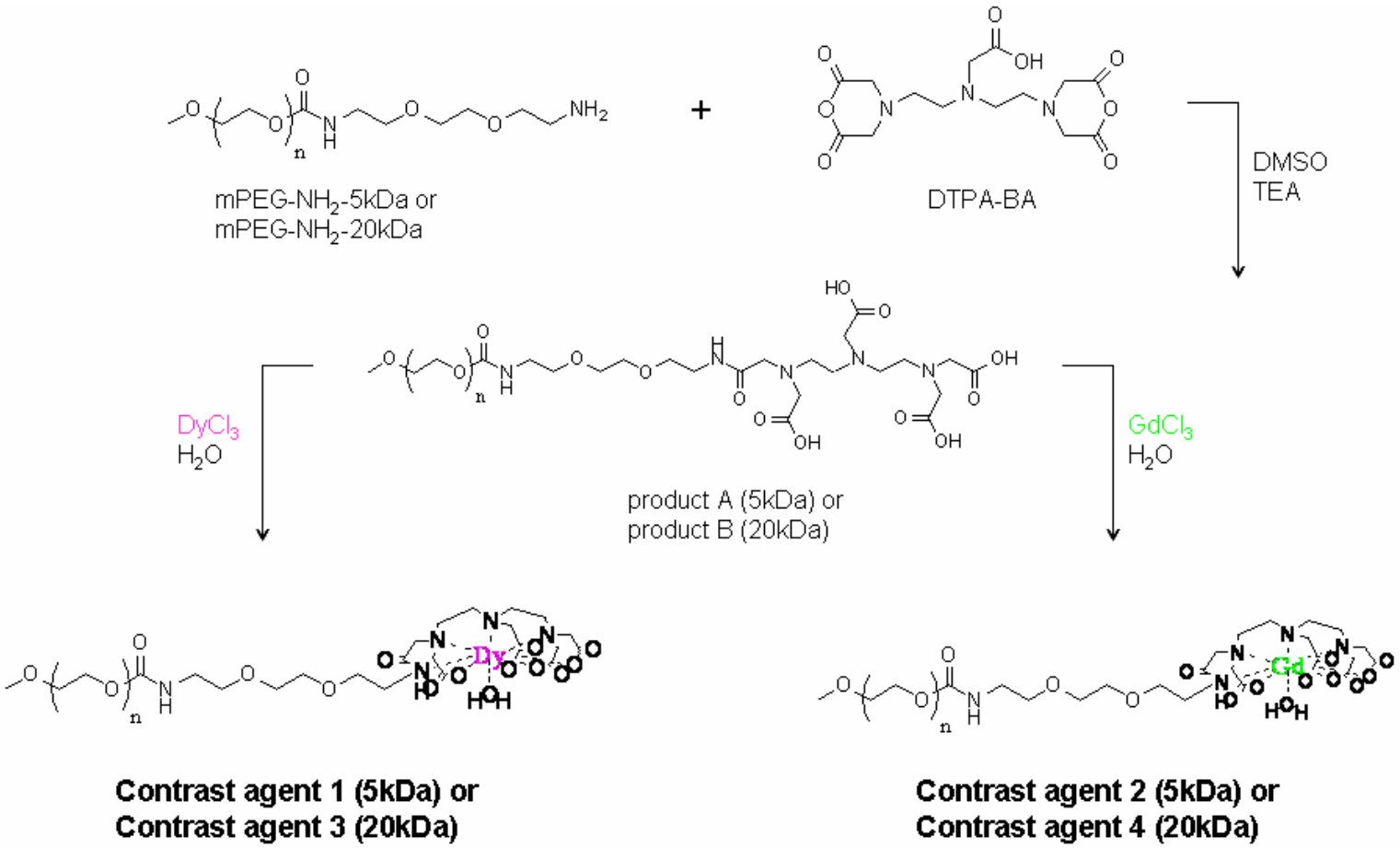

Figure 3-1 Synthesis of contrast agents 1 and 2 with mPEG-NH_2-5kDa and contrast agents 3 and 4 with mPEG-NH_2-20kDa. 1 and 3 contain Dy while 2 and 4 contain Gd.

TNBS assay

A TNBS assay [36] was performed to determine the reaction yields of products A and B (Figure 3-1). Four samples were measured consisting of products A and B as well as the starting materials, mPEG-NH_2-5kDa and mPEG-NH_2-20kDa. All samples were prepared at a concentration of 1mg per 1mL (0.157mM for product A, 0.051mM for product B, 0.167mM for mPEG-NH_2-5kDa, and 0.052mM for mPEG-NH_2-20kDa) in 0.1M sodium bicarbonate (pH 8.5) buffer solution. Six other samples were prepared, one as a control and the others to create a standard curve by which to compare the experimental data. The control was 1mL of buffer (0.1M sodium bicarbonate, pH 8.5) and the five standard samples were made from Gly-Pro starting at 0.05mM then diluted by half to get a range of concentrations (0.05mM, 0.025mM, 0.0125mM, 0.00625mM, and 0.003125mM).

A stock solution (prepared fresh) of TNBS was prepared at a concentration of 0.01% (w/v) in 0.1M sodium bicarbonate buffer (pH8.5) and 0.5mL of this stock solution was added to each of the 10 samples. The samples were then incubated at 37ºC for 2 hours. Before measuring the UV absorbance of each sample, the samples were diluted by 10 fold in sodium bicarbonate buffer (pH8.5). The absorbance at 345nm for each sample was then measured and recorded. The standard values were graphed on a chart with the x-axis labeled concentration and the y-axis labeled absorbance. The linear model determined by this data was then used to calculate the percent yield of the reactions for products A and B.

T_2 measurements

All T_2 measurements were taken on a 400MHz Varian NMR machine using a Carr-Purcell Meiboom-Gill T2 (C-P-M-G-T2) pulse sequence at room temperature in the standard NMR tubes. For the T_2 measurements of contrast agent 1, 63mg of 1 was added to 1mL of PBS buffer (0.2M, pH 7.4) in an eppendorf tube. The tube was vortexed for 30 seconds and then a 0.5mL aliquot was removed and placed in a NMR tube. 0.5mL of buffer was added to the remaining solution to dilute it by half. This procedure was repeated for a total of 5 NMR samples (sample concentrations: 9.7mM, 4.85mM, 2.425mM, 1.2125mM, 0.60625mM). The same procedure as above was followed to measure the T_2 of contrast agents 2 (sample concentrations: 6.3mM, 3.15mM, 1.575mM), 3 (sample concentrations: 2mM, 1mM, 0.5mM, 0.25mM, 0.125mM), and 4 (sample concentrations: 1.3mM, 0.65mM, 0.325mM, 0.1625mM, 0.08125mM) with 41mg, 52mg and 43mg as the starting amounts, respectively. The T_2 data was collected and plotted on a graph using the following equation:

$$1/T_{2obs} = R_2[c] + 1/T_{2H2O} \qquad \text{(Equation 3-1)}$$

Where T_{2obs} is the T_2 measured from the sample, [c] is the concentration, R_2 is the slope of this line, and T_{2H2O} is the T_2 of water (which is 3s at 9.4T).

T_2 pulse sequence

To measure the T_2 of all the samples in this work the C-P-M-G-T2 pulse sequence was used on a 400MHz Varian NMR machine. This sequence utilizes a 90º pulse followed by a series of 180º pulses that are separated by an array of times set by the user to achieve full decay of the signal. Figure 3-2 is a schematic that illustrates the sequence of pulses where pw, p1, and d1 are set by the machine, and d2 is set at 4ms.

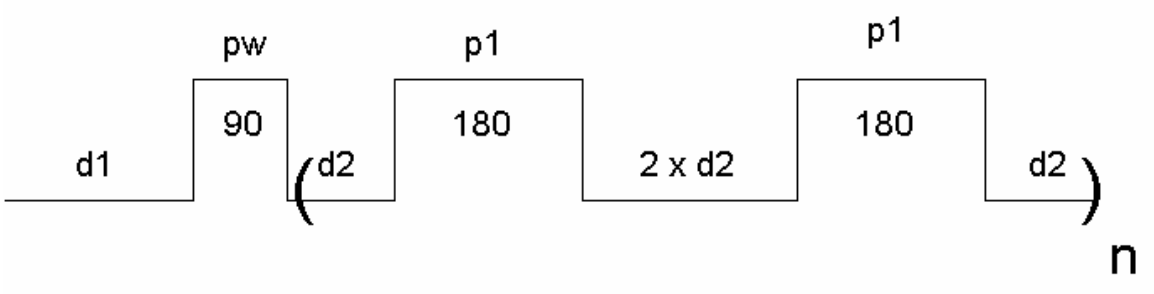

Figure 3-2 CPMGT2 signal pulse sequence where n represents the number of times this section of the sequence is repeated

For all samples the array was set to give enough time for full signal decay and an error margin of less than 10%.

Synthesis of contrast agent 5

Synthesis of C

To make C (Figure 3-3) [37], 180mg (0.82mmol) of aldrithiol was dissolved in a solution of 20mL methanol and 0.8mL acetic acid in a 100mL round bottom flask equipped with a magnetic stir bar. In a separate 50mL round bottom flask, 50mg (0.65mmol) of cysteamine was dissolved in 10mL methanol. The mixture was then added drop-wise with stirring to the solution containing aldrithiol over 30 minutes for a final reaction volume of 30mL methanol and then sealed with a rubber septum. The mixture was allowed to react for 4 hours at room temperature. To purify C from the by-products and reactants, silica gel chromatography was performed using 7g of silica gel. The reaction

mixture was concentrated by rotary vacuum distillation to about 1mL and was loaded on the silica gel column. To elute the reactants and the by-product, 100% ethyl acetate was used and the collected fractions were discarded. The product was then eluted using a MeOH/TEA (80/20) mixture and the fractions containing the product (determined by thin layer chromatography) were collected and vacuum dried for 48 hours. The final product was analyzed by NMR and UV-Vis.

The reaction yield determined by UV-Vis was calculated by measuring the UV absorbance of the by-product (pyridyl-2-thione) in the reaction of C with DTT. For the UV-Vis assay, a sample of C (1.5mg) was dissolved in 1mL PBS buffer (0.2M, pH 7.4) and buffer alone was used as the control. UV spectra were taken of each sample before and after the addition of DTT (0.5mM final concentration) and the change in absorbance at 340nm were measured for each sample. The control had little change in absorbance at 340nm, while the sample containing C had a significant increase in absorbance at 340nm. The concentration of pyridyl-2-thione was determined using the following equation and compared to the theoretical concentration to obtain the percent yield.

$$A = \varepsilon[c]d \qquad \text{(Equation 3-2)}$$

Where A is the UV absorbance of the sample, ε is the extinction coefficient (in this case $\varepsilon = 8.08 \times 10^3\ M^{-1}cm^{-1}$), [c] is the concentration of the sample (in M), and d is the light-path distance (in this case it's equal to 1cm).

Synthesis of D

In a 5mL round bottom flask, 100mg (4.7μmol) of mPEG-SH-20kDa was dissolved in 1mL PBS buffer (0.1M, pH 9). After the polymer was fully dissolved, 1.3mg (7μmol) of C

was added to the reaction flask and sealed with a rubber septum. The reaction was monitored by removing aliquots of the reaction hourly and talking their UV spectrum. To determine the amount of pyridyl-2-thione, a reaction by-product, present in the solution, the change in the change in absorbance at 340nm was monitored. After 3 hours, the reaction had come to completion. An additional 0.43mg of product C was added to the reaction flask and after another hour had elapsed, no further increase in the absorbance at 340nm was detected, indicating that all the mPEG-SH-20kDa had reacted. The reaction mixture was purified by dialysis (3,500 molecular weight cut-off; 4 water changes for a total of 10L) for 24 hours and then freeze-dried for an additional 24 hours.

Synthesis of E

All of the material recovered from the freeze-drying process in the synthesis of D (see above) was re-dissolved in 1mL DMSO and 10μL (0.07mmol) TEA in a scintillation vial equipped with a magnetic stir bar. 4mg (0.011mmol) of DTPA-BA was dissolved in 1mL DMSO and then added drop-wise to the vial containing D, for a total reaction volume of 2mL DMSO. The reaction vial was sealed with the scintillation vial cap and allowed to react for 24hours at room temperature. After 24 hours elapsed, 20mg (0.074mmol) of $DyCl_3$ was added to the reaction vial. The mixture was resealed and stirred for 4 hours at room temperature, after which it was placed in a dialysis bag (3,500 molecular weight cut-off; 4 water changes for a total of 10L) and dialyzed for 24 hours against deionized water. The product was then removed from the dialysis bag and freeze-dried for 24 hours. A total of 54mg (54% of the original mPEG-SH-20kDa weight) was recovered from the freeze-dryer.

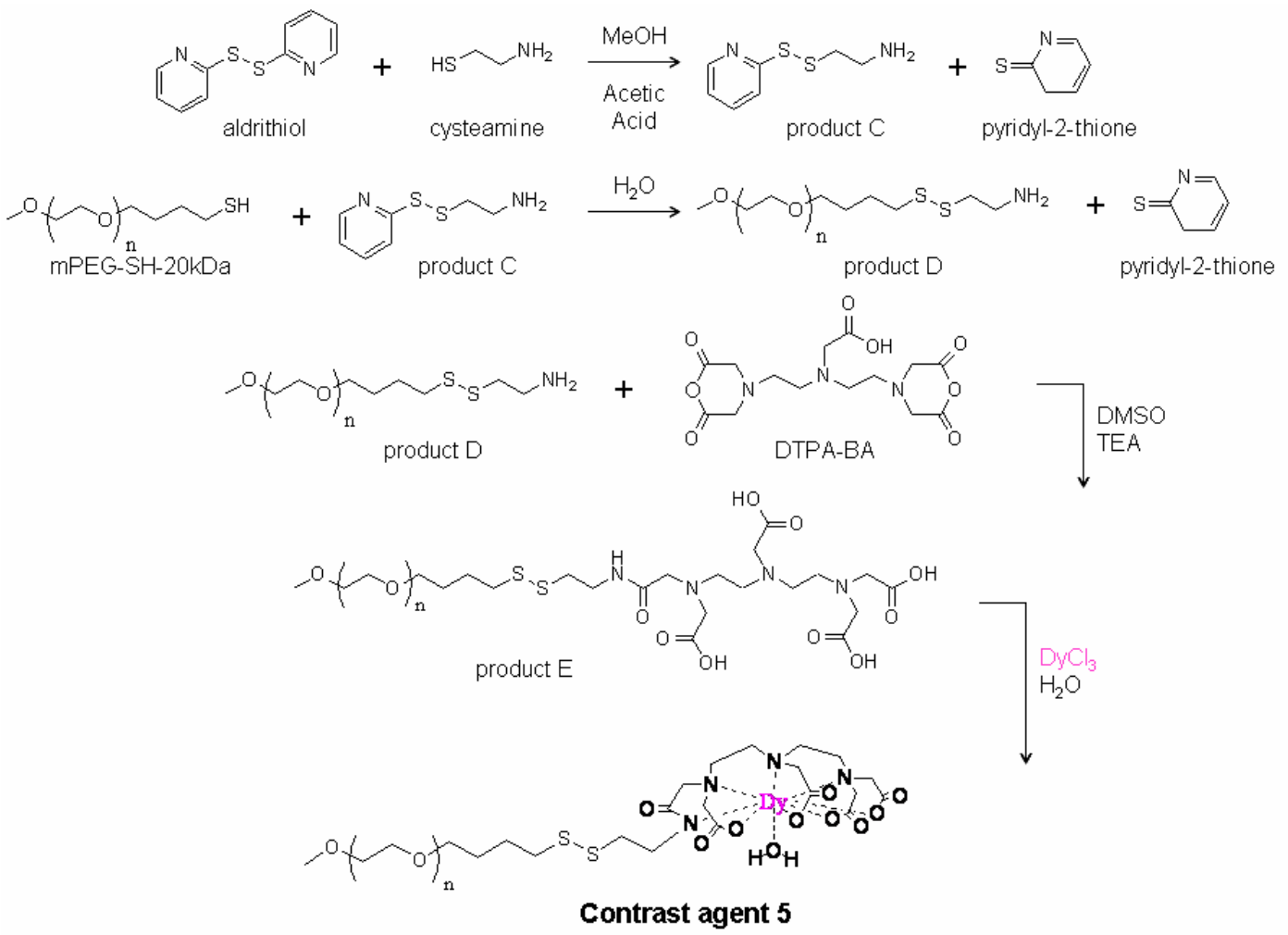

Figure 3-3 Synthesis of contrast agent 5.

Synthesis of contrast agent 6

To synthesize contrast agent 6 (Figure 3-4), 250mg (0.012mmol) of mPEG-OPSS-20kDa was dissolved in 2.5mL deionized water in a scintillation vial equipped with a magnetic stir bar. In an eppendorf tube, 11.2mg (0.15mmol) of cysteamine was dissolved in 0.5mL deionized water and then added to the scintillation vial for a total reaction volume of 3mL. Over the course of the reaction the amount of pyridyl-2-thione, the reaction by-product was monitored by UV (absorbance at 340nm) to determine completion time and yield. The reaction mixture was then placed in a dialysis bag (3,500 molecular weight cut-off; 5 water changes for a total of 12L) and dialyzed for 24 hours against deionized water. After dialysis, the solution recovered from the dialysis bag was placed in a scintillation vial and freeze-dried for 24 hours. 230mg (92% of original PEG amount) of a white, fluffy powder was recovered from the freeze-drying process (product F).

The next step in the synthesis of 6 was the reaction of 230mg (0.01mmol) of product F with 16mg (0.045mmol) of DTPA-BA in a DMSO/TEA (3mL/10μL) solution. The reaction was stopped after 24 hours and then placed in a dialysis bag (3,500 molecular weight cut-off; 4 water changes for a total of 10L) and dialyzed against deionized water for an additional 24 hours for purification. The reaction solution, containing product G, was then removed from the dialysis bag and placed in a scintillation vial equipped with a magnetic stir bar. 40.7mg (0.15mmol) of $DyCl_3$ was added directly to the reaction solution and was allowed to stir for 24 hours. The reaction solution was then again dialyzed against deionized water (3,500 molecular weight cut-off; 4 water changes for a

total of 10L) to remove any unchelated $DyCl_3$ for 24 hours. The solution was then freeze-dried to remove water from the final product.

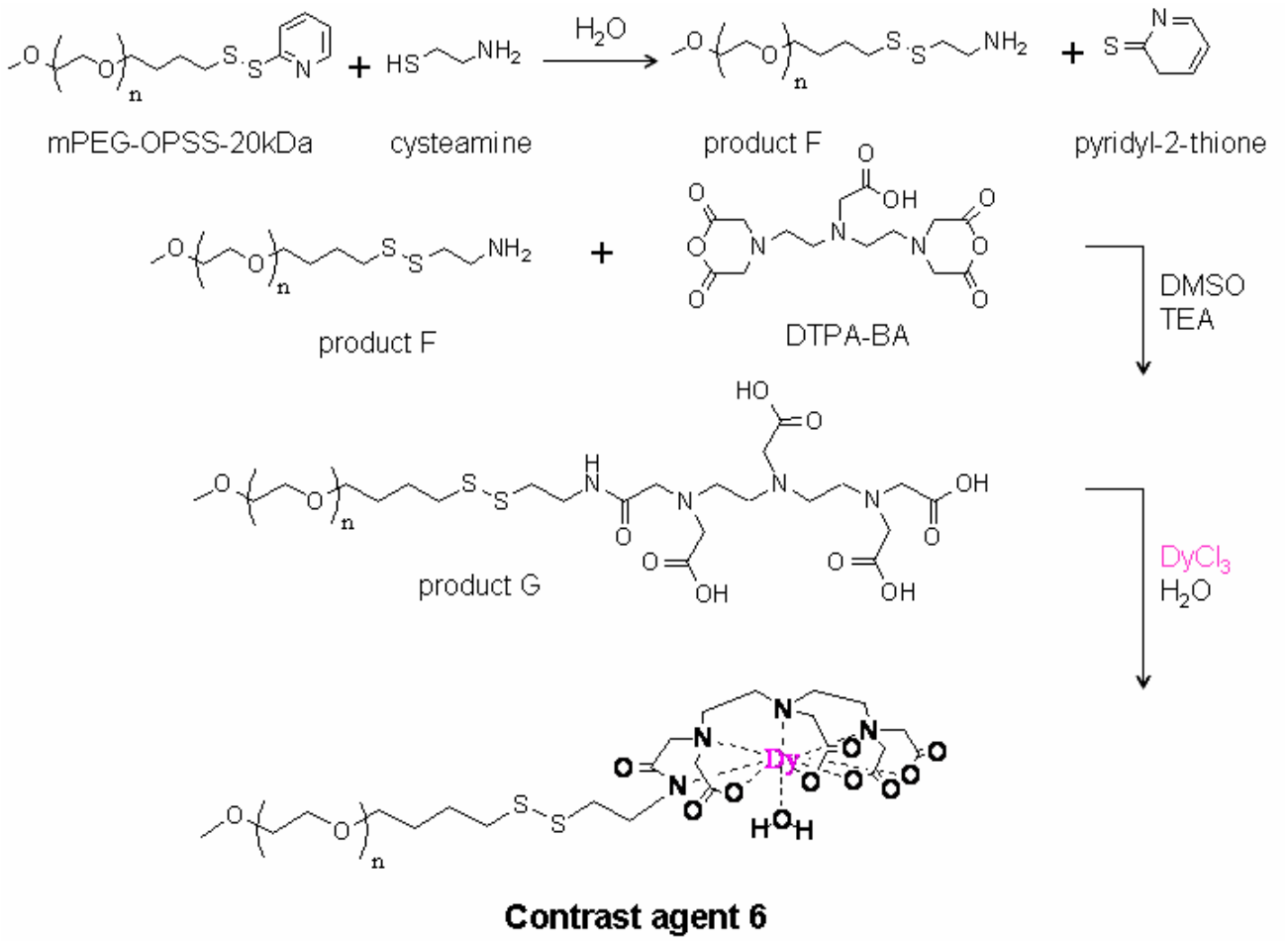

Figure 3-4 Synthesis contrast agent 6.

GSH experiments with 5

To measure the change in T_2 of 5 in the presence of GSH, 5 samples were made each with a concentration of 0.75mM in PBS buffer (0.2M, pH 7.4). A total of 40mg of 5 was weighed out and added to 2.5mL of PBS buffer in a scintillation vial, vortexed for 30 seconds and then 0.5mL aliquots were taken and placed in NMR tubes. A control of 0.75mM of 3 was used (10.2mg in 0.638mL PBS buffer) to see if GSH could elicit a change in T_2 without the disulfide exchange reaction occurring. A solution of GSH was made (46mg/0.5mL) in PBS buffer to give a final concentration in the NMR tubes of 3mM (4x the concentration of 5). The T_2 measurements were taken on a 9.4 Varian NMR machine at room temperature using a C-P-M-G-T2 pulse sequence. The T_2 measurements of all 6 samples (5 experimental and 1 control) were taken and then 5µL of the GSH solution was added to each sample (reaction temperature was 25ºC). The T_2 measurements were then repeated at 4 hours and then at 31 hours after the addition of GSH.

GSH experiments with 6

To measure the change in T_2 of 6 in the presence of GSH, 3 samples were made each with a concentration of 0.46mM in PBS buffer (0.2M, pH 7.4). A total of 15mg of 6 was weighed out and added to 1.5mL of PBS buffer in a scintillation vial, vortexed for 30 seconds and then 0.5mL aliquots were taken and placed in NMR tubes. A separate solution of 6 was used (5mg in 0.5mL PBS buffer; 0.46mM) as a control to determine the behavior of 6 under the same conditions without the addition of GSH. A solution of GSH was made (37mg/0.5mL) in PBS buffer to give a final concentration in the NMR tubes of

1.84mM (4x the concentration of 6). The T_2 measurements were taken on a 9.4 Varian NMR machine at room temperature using a C-P-M-G-T pulse sequence. The T_2 measurements of all 4 samples (3 experimental and 1 control) were taken and then 10μL of the GSH solution was added to each sample except the control and then all samples were placed in a water bath at 37ºC. The T_2 measurements were then repeated at 5 hours and then at 24 hours after the addition of GSH.

CHAPTER 4

RESULTS

Reaction yields of contrast agents 1, 2, 3, and 4

A TNBS assay was performed on products A (5kDa) and B (20kDa) as well as the starting materials (mPEG-NH_2-5kDa and mPEG-NH_2-20kDa) to determine the reaction yields with DTPA-BA. Using the standard curve generated by the absorbance data of the various concentrations of Gly-Pro, it was determined that the synthesis of product A (Figure 3-1) had a yield of 97% and product B (Figure 3-1) had a yield of 90.2%.

Interestingly, it was also found that the starting materials had low free amine content by weight; mPEG-NH_2-5kDa had 24% free amine content by weight and mPEG-NH_2-20kDa had 33%. This means that there was a substantial amount of water and PEG chains without a free amines contributing to the bulk weight of the starting materials. This extra content would not contribute to the synthesis. The products cannot be purified from the extra PEG and it will remain in the system throughout the T2 measurements, but would not contribute to the T2 values measured.

All reactions involving $DyCl_3$ or $GdCl_3$ were assumed to result in 100% chelation due to the high affinity of DTPA to the metal ions.

T_2 measurements of contrast agents 1 and 3

This section will present the results obtained from the T_2 measurements taken of contrast agents 1 and 3. These polymers contain Dy and therefore should have a lowering effect on the T_2 of the solution versus the T_2 of water, which is 3s at 9.4T. All measurements were taken in a PBS buffer (0.2M, pH 7.4) at room temperature on a 9.4T Varian NMR machine. Each contrast agent was measured at 5 concentrations for statistical purposes.

The following tables (Table 4-1 and Table 4-2) gives the concentrations measured, T_2 results, measurement errors, and the 1/ T_2 used for graphing purposes for contrast agents 1 and 3.

Table 4-1 T_2 data for contrast agent 1
PEG-DTPA-Dy-5kDa (1)

Concentration (mM)	T_2 (s)	Error	1/ T_2 (s^{-1})
9.7	0.01808	0.000125	55.30973
4.85	0.03037	0.00013	32.92723
2.425	0.07046	0.001486	14.19245
1.2125	0.1509	0.008728	6.626905
0.60625	0.2152	0.01497	4.64684

Table 4-2 T_2 data for contrast agent 3
PEG-DTPA-Dy-20kDa (3)

Concentration (mM)	T_2 (s)	Error	1/ T_2 (s^{-1})
2	0.04141	0.001005	24.14876
1	0.09421	0.002654	10.61458
0.5	0.1915	0.009936	5.221932
0.25	0.3292	0.01809	3.037667
0.125	0.4459	0.03237	2.242655

To view these results graphically, the following equation was applied to the data:

$$1/T_{2obs} = R_2[c] + 1/T_{2H2O} \quad \text{(equation 4-1)}$$

Where T_{2obs} (s) is the T_2 measured from the sample, [c] is the concentration (mM), R_2 is the slope of this line ($mM^{-1}s^{-1}$), and T_{2H2O} is the T_2 of water (T_2 of water at 9.4T is 3s). Since T_2 is concentration dependent, R_2 is the measure of the effectiveness of the contrast agent to lower T_2 and the value determined by this experiment.

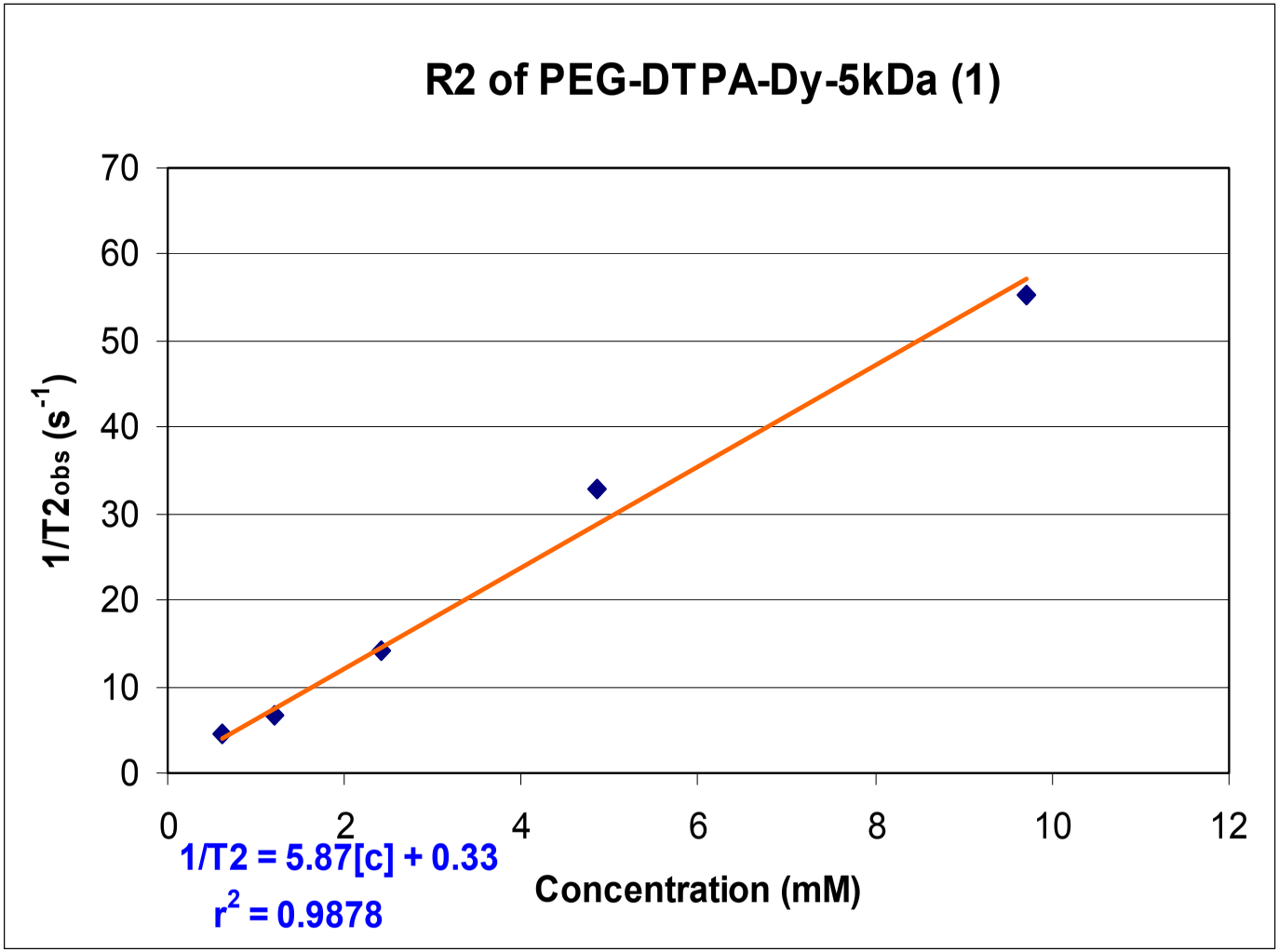

Figure 4-1 Concentration (mM) versus $1/T_{2obs}$ (s^{-1}) to calculate the R_2 value of 1, which is 5.87 $mM^{-1}s^{-1}$ with a r^2 value of 0.9878 (linear fit).

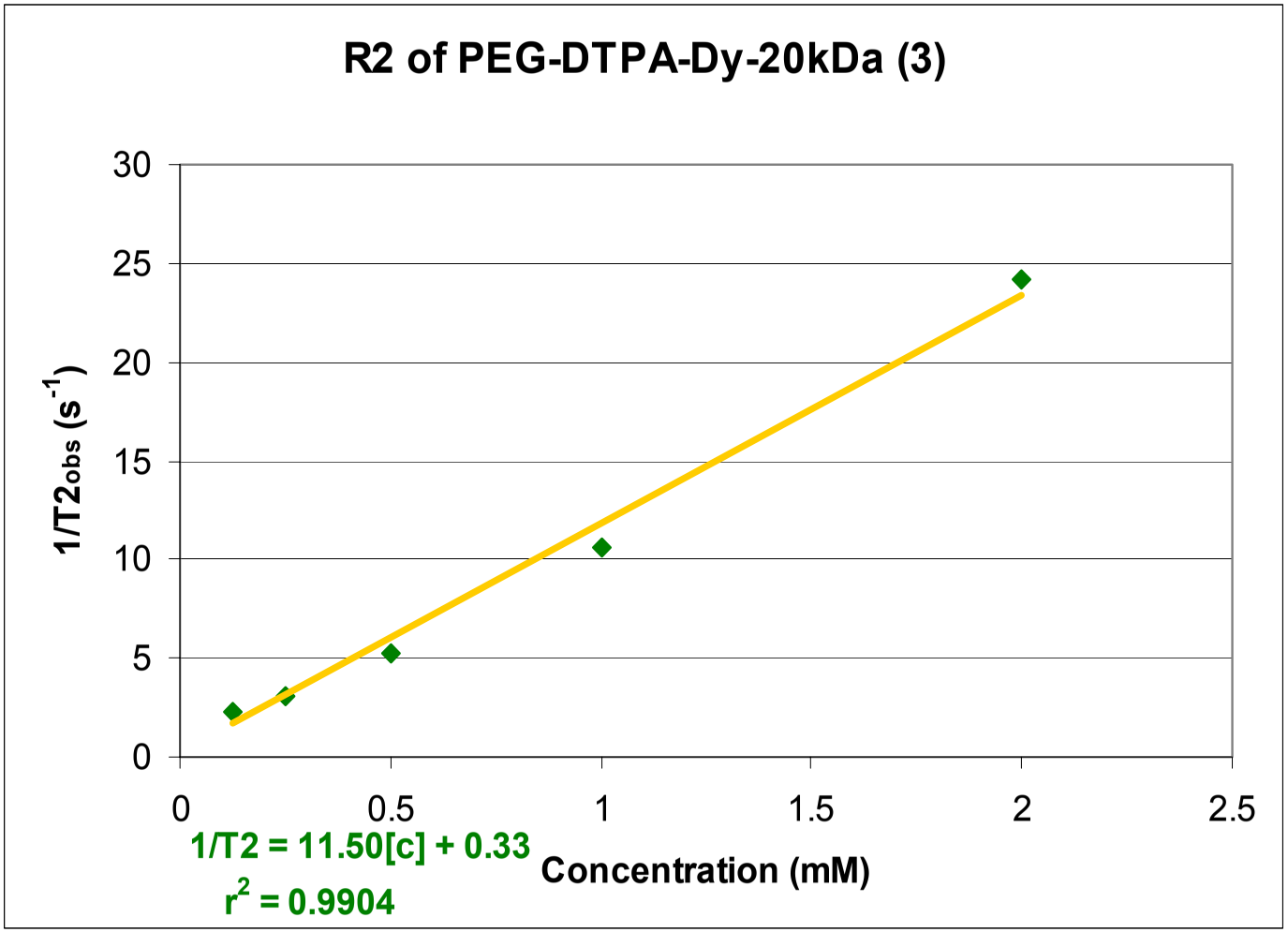

Figure 4-2 Concentration (mM) versus $1/T_{2obs}$ (s^{-1}) to calculate the R_2 value of 3, which is 11.50 $mM^{-1}s^{-1}$ with a r^2 value of 0.9904 (linear fit).

It can be seen from Figure 4-1 that the R_2 of 1, which has a molecular weight of about 5kDa, is 5.87$mM^{-1}s^{-1}$ with a linear fit of 0.9878. The R_2 of 3, which has a molecular weight of about 20kDa, is 11.50$mM^{-1}s^{-1}$ with a linear fit of 0.9904 (Figure 4-2). With r^2 values close to 1, it can be assumed that the data fits the equation well and the calculated R_2 values are very close to the actual solution R_2 values.

T_2 measurements of contrast agents 2 and 4

The results obtained from the T_2 measurements of 2 and 4 are presented in this section. These contrast agents contain Gd and should therefore lower T_2 as compared to pure water (which at 9.4T is 3s). All measurements were taken in a PBS buffer (0.2M, pH 7.4) at room temperature on a 9.4T Varian NMR machine. Each contrast agent was measured at several concentrations for statistical purposes.

For comparison to the results obtained with the contrast agents containing Dy, the same experiments were performed on 2 and 4. The following Tables (Tables 4-3 and 4-4) display the raw data collected during the T_2 measurements and the Figures (Figures 4-3 and 4-4) display the data using equation 4-1 to determine the R_2 values for 2 and 4.

Table 4-3 T_2 data for contrast agent 2
PEG-DTPA-Gd-5kDa (2)

Concentration (mM)	T_2	Error	$1/T_2$
6.3	0.0246	0.000142	40.65041
3.15	0.06428	0.00044	15.55694
1.575	0.1241	0.001236	8.058018

Table 4-4 T_2 data for contrast agent 4
PEG-DTPA-Gd-20kDa (4)

Concentration (mM)	T_2	Error	$1/T_2$
1.3	0.1052	0.000788	9.505703
0.65	0.171	0.003369	5.847953
0.325	0.374	0.01472	2.673797
0.1625	0.5436	0.04344	1.839588
0.08125	0.6137	0.05368	1.629461

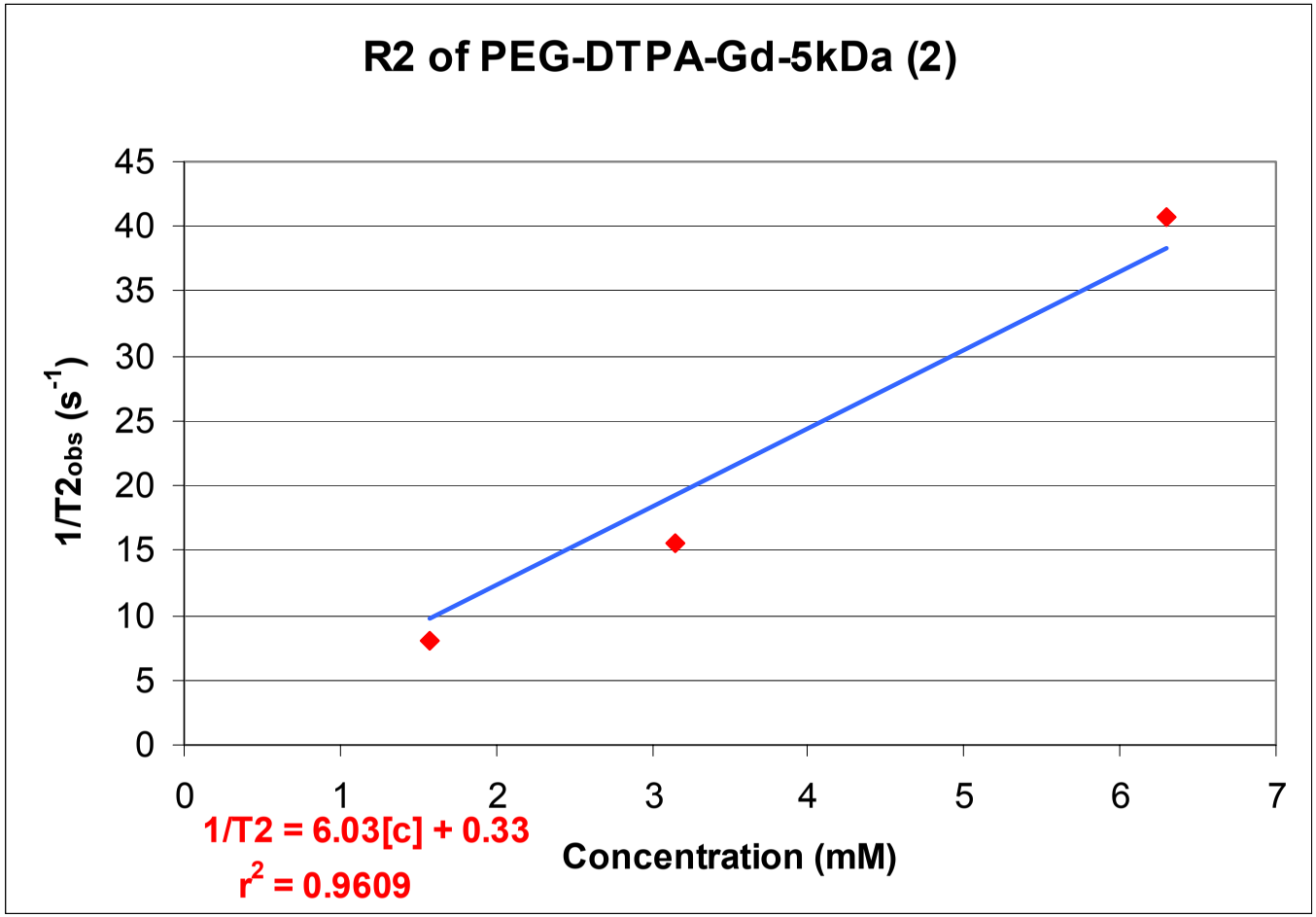

Figure 4-3 Concentration (mM) versus $1/T_{2obs}$ (s^{-1}) to calculate the R_2 value of 2, which is 6.03 $mM^{-1}s^{-1}$ with an r^2 value of 0.9609 (linear fit).

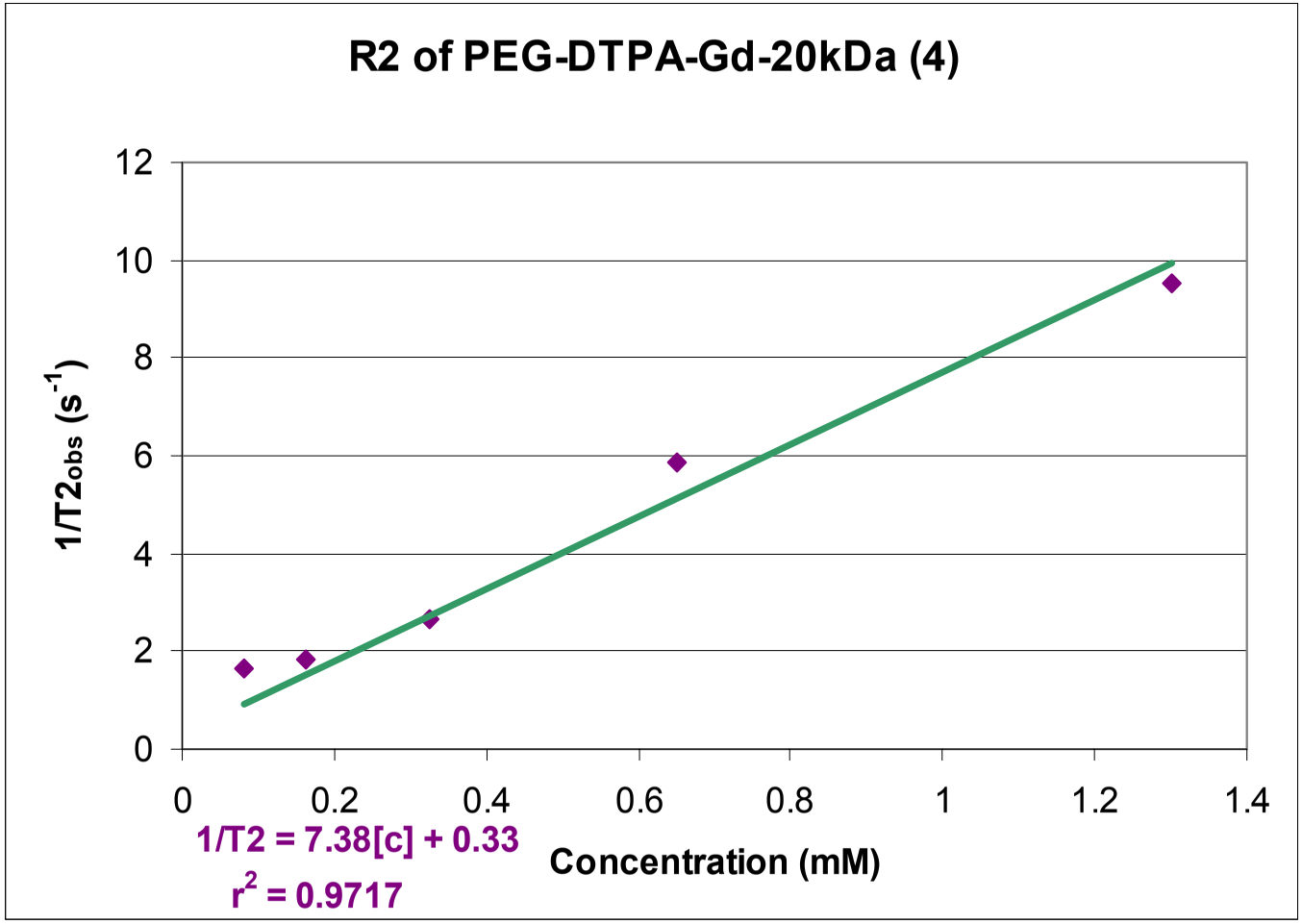

Figure 4-4 Concentration (mM) versus $1/T_{2obs}$ (s^{-1}) to calculate the R_2 value of 4, which is 7.38 $mM^{-1}s^{-1}$ with an r^2 value of 0.9717 (linear fit).

Results of the synthesis of contrast agent 5

The first step in the synthesis pathway of contrast agent 5 (Figure 3-3) is to make product C. Since this product is a small-molecule, several methods were employed to determine if it was made properly and its reaction yield. The first evidence of product C was seen in a NMR spectrum (see Figure 4-5). All proton peaks are present and at the correct ratios.

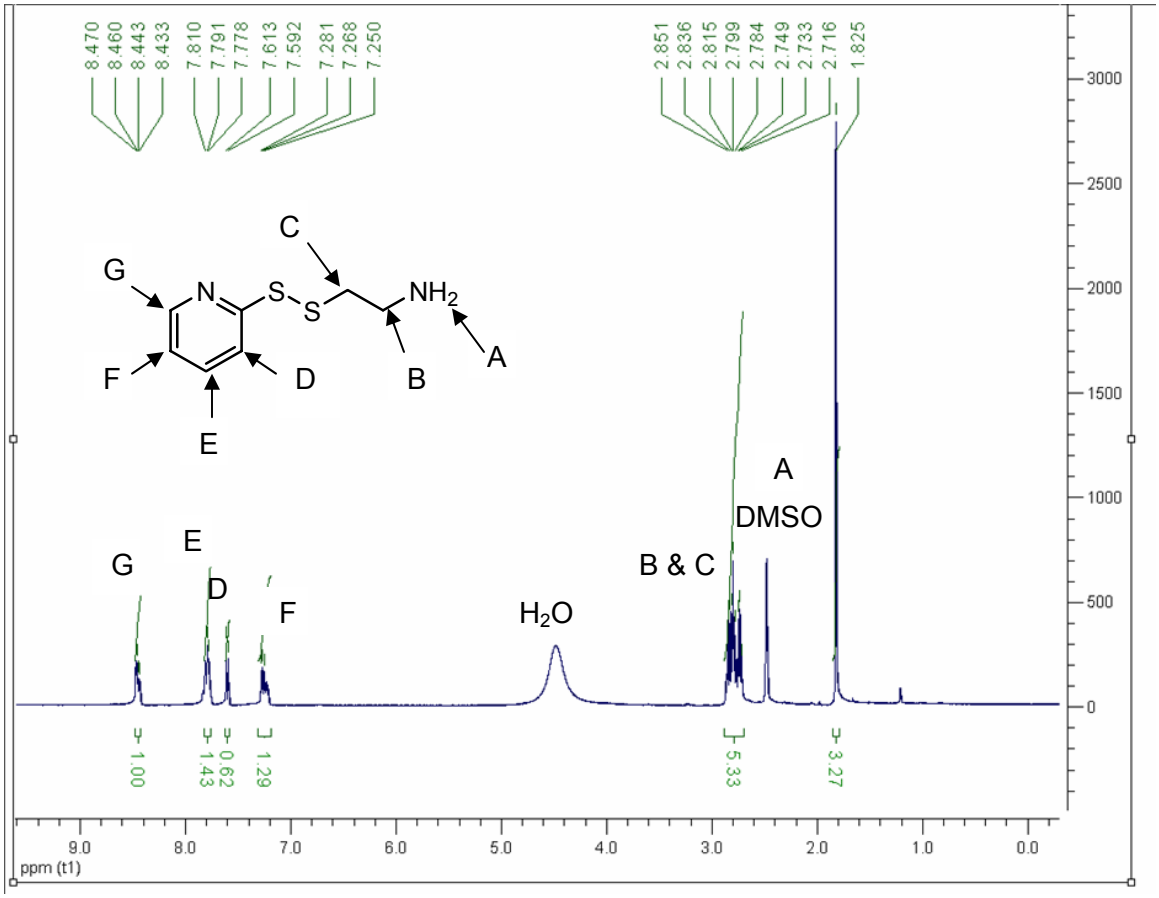

Figure 4-5 NMR spectrum of product C in deuterated DMSO, where A-G indicate their respective protons

The reaction yield of C was calculated from two methods, by UV-Vis absorbance data and by weight (comparing actual weight to theoretical weight). The final reaction yield was 78% based on weight and 87.5% based on UV-Vis.

The reaction for the second step (product D) in the synthesis of contrast agent 5 was monitored by the UV absorbance of the by-product, pyridyl-2-thione. This reaction was finished after 5 hours and even with further addition of product C, no increase in absorbance was measured by UV.

The final steps in the synthesis of contrast agent 5 were not monitored. The reaction of product D with DTPA-BA was assumed to have about 90% reaction yield since this is a similar reaction to the one in the synthesis pathway of contrast agents 1, 2, 3, and 4. The chelation of Dy^{3+} to DTPA was also assumed to have a reaction yield of 100%, due to the high affinity of DTPA to the metal ion.

GSH T_2 experiments with contrast agent 5

To determine if the presence of GSH can be detected by MRI using contrast agent 5, five samples (0.75mM each) of contrast agent 5 were prepared in PBS buffer (0.2M, pH 7.4) and pipetted into NMR tubes. A control was prepared using contrast agent 3 at the same concentration (0.75mM) in PBS buffer (0.2M, pH 7.4) and was also placed in a NMR tube. This control was chosen to determine if GSH changes the T_2 of the solution.

Table 4-5 shows the T_2 data collected for each sample (A-D) and the control. The average and standard deviation were calculated for samples A-D without the control data. Figure 4-6 illustrates the data in a bar graph, with markers indicating the standard deviation for each. The average T_2 for the initial data is 0.3189s, then 5 hours after the addition of GSH the average T_2 drops to 0.2784s, which may indicate an unstable system. After 31.5 hours, the system recovers back to 0.3451s. Another indication of system instability is the relatively large standard deviation for the initial time point, which is ±18% versus ±1% for the 5 hour time point and ±5% for the 31.5 hour time point.

The control for this experiment also drifted with time. This may be due to a small alteration of T_2 by GSH and some system instability with contrast agent 3. It changed from the initial T_2 measurement of 0.1226s to 0.1583s at 5 hours to 0.1838s at 24 hours for a total change of 50%.

Table 4-5 T_2 data for contrast agent 5 with GSH

	Time After Addition of GSH		
Sample	Initial (no GSH) T_2 (s)	T_2 (s) at 5hrs	T_2 (s) at 31.5hrs
A	0.3458	0.2743	0.3295
B	0.3657	0.2757	0.3244
C	0.2422	0.2751	0.3614
D	0.273	0.2894	0.3677
E	0.3676	0.2773	0.3424
Contol	0.1226	0.1583	0.1838
Average	**0.3189**	**0.2784**	**0.3451**
Std. dev.	**0.0576**	**0.0063**	**0.0191**

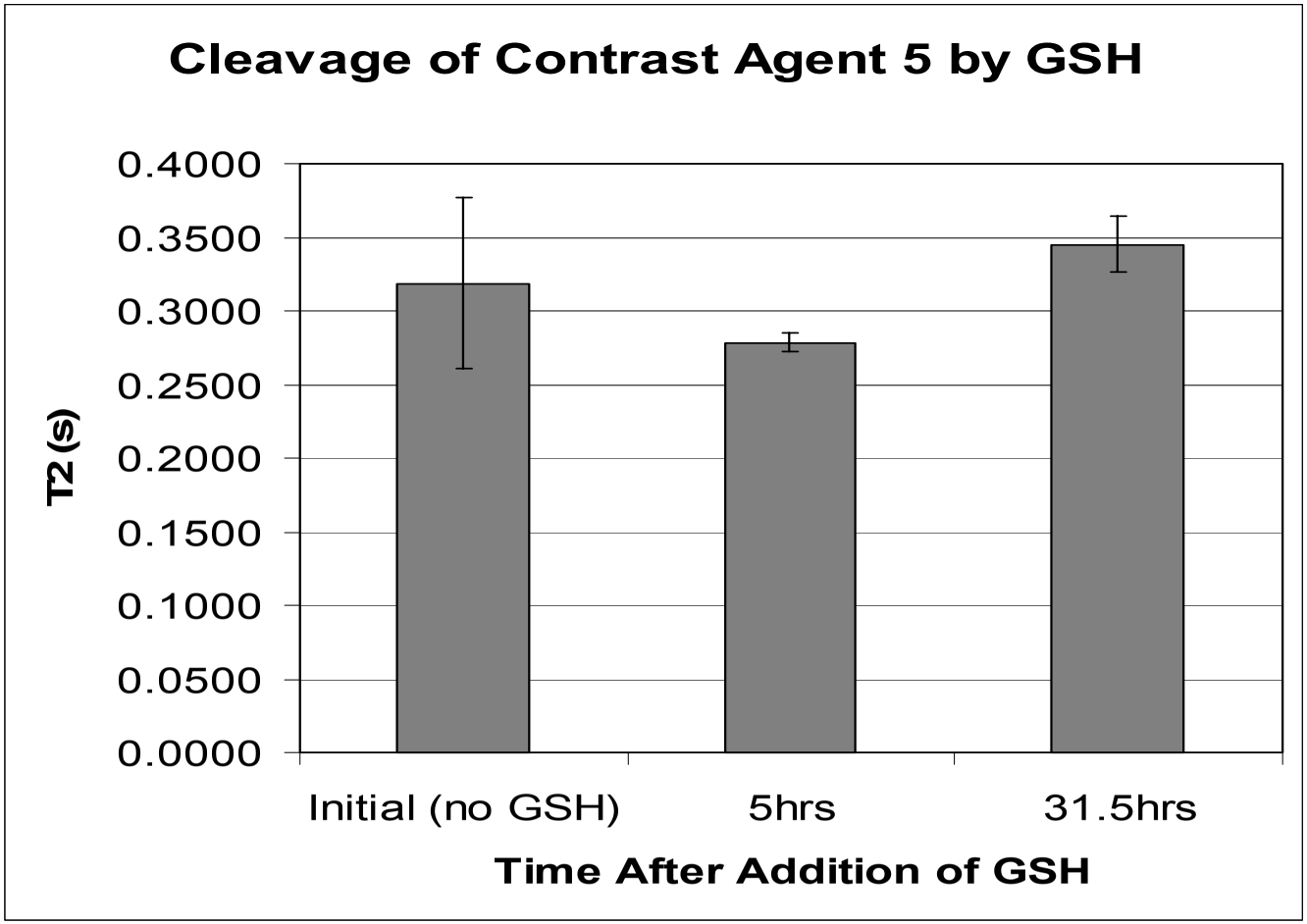

Figure 4-6 The average T_2 data for contrast agent 5

Results of the synthesis of contrast agent 6

The fist step in the synthesis of contrast agent 6 was monitored by the increase in UV absorbance of the by-product, pyridyl-2-thione, that is formed during the reaction of cysteamine with mPEG-OPSS-20kDa. From this data it was determined that the reaction had come to completion in 4 hours and had a 76% yield. As in the synthesis of contrast agent 5, the final two steps in the synthesis of contrast agent 6 were assumed to have a high reaction yield and were not measured.

GSH T_2 experiments with contrast agent 6

The T_2 experiment with contrast agent 6 and GSH was conducted in a similar fashion to the one discussed above for contrast agent 5. Three samples (0.46mM each) of contrast agent 6 were made in PBS buffer (0.2M, pH 7.4) and pipetted into NMR tubes. A separate solution of 6 at the same concentration (0.46mM) in a NMR tube was used as a control. This control would not have GSH added to the solution to monitor the behavior of contrast agent 6 in solution over the duration of the experiment.

Table 4-6 lists the T_2 data collected during the experiment for samples A-C and the control. The average and standard deviation were calculated for samples A-C excluding the control data. The bar graph (Figure 4-7) illustrates the average percent change between the initial data and the 24hr time point data. At the initial time-point, before the addition of GSH, the average T_2 for the samples is 0.1889s ±8%. Five hours after the addition of GSH to each sample solution, the T_2 of the samples increased to an average

of 0.2763s ±11% and then at 24hours (19 hours later) the average T_2 was 0.3230s ±7%. These results follow the expected trend of an increasing T_2 with time after the addition of GSH.

The control (one sample) in this experiment consisted of contrast agent 6 in buffer that was treated under the same conditions as the other three samples, only without the addition of GSH to the solution. The control had an initial T_2 measurement of 0.3735s and then rose to 0.4317s at 5 hours and 0.4561s at 24 hours. This was a total change of 22%.

Table 4-6 T_2 data for contrast agent 6 with GSH

	Time After Addition of GSH		
Sample	Initial (no GSH) T_2 (s)	T_2 (s) at 5hrs	T_2 (s) at 24hrs
A	0.1803	0.2566	0.3156
B	0.1805	0.261	0.3473
C	0.206	0.3112	0.3062
Control	0.3735	0.4317	0.4561
Average	**0.1889**	**0.2763**	**0.3230**
Standard deviation	**0.0148**	**0.0303**	**0.0215**

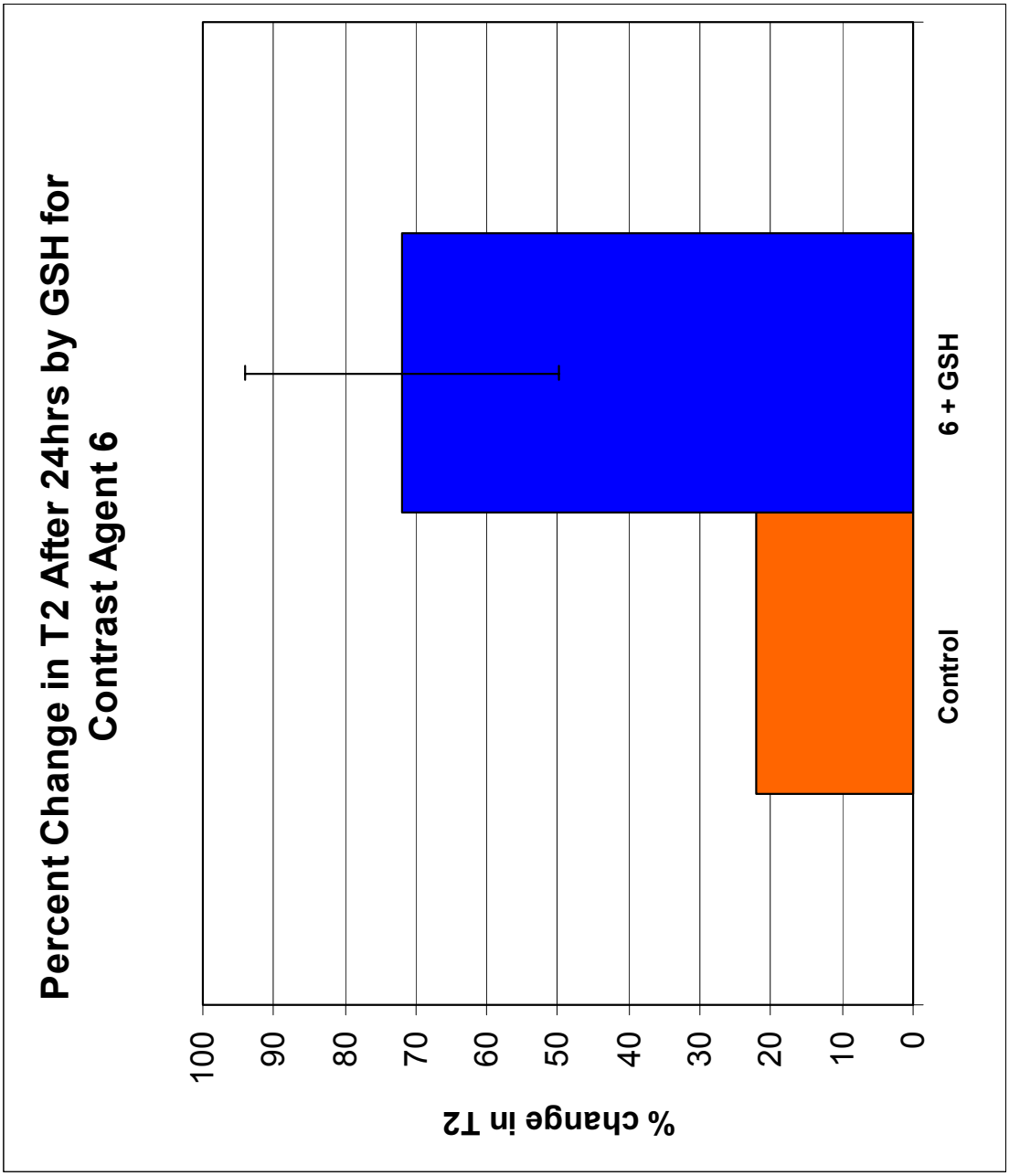

Figure 4-7 Percent change in T_2 for contrast agent 6

CHAPTER 5

DISCUSSION

In this work, the effect of covalently bonding two molecular weight PEGs to DTPA, a Dy ligand, was studied to determine if the MRI contrast ability of Dy could be enhanced. From Figures 4-1 and 4-2, it can be seen that as the molecular weight of the PEG chain increases from 5kDa to 20kDa, the R_2 of the contrast agents doubles. The R_2 of contrast agent 1, which has a molecular weight of 5kDa, is 5.87$mM^{-1}s^{-1}$ and the R_2 of contrast agent 3, which has a molecular weight of 20kDa, is 11.50$mM^{-1}s^{-1}$. Literature values for the R_2 of DTPA-Dy are 0.5 $mM^{-1}s^{-1}$ at 7.05T and 1.5 $mM^{-1}s^{-1}$ at 11.75T (it can be estimated that at 9.4T the R_2 of DTPA-Dy would lie within this range), which is significantly less than the values resulting from this work [10]. This indicates that by increasing the chain length of the PEG covalently bonded to DTPA-Dy, the MRI contrast ability of the Dy is enhanced at 9.4T.

The enhancement of the contrast ability of DTPA-Dy through pegylation is due to a combination of slower water exchange rates, better susceptibility, and a slower rotational correlation time, which are all beneficial in a Dy-based contrast agent. From the literature it is seen that PEG and amide bonds (contrast agents 1 and 3 each have one amide bond) can successfully slow the exchange rate of the water coordinated in the DTPA-Dy system [5, 10, 11, 27]. It is also possible that DTPA-Dy contrast agents with large molecular weight PEGs grafted to them have an increased susceptibility effect due to the large hydrodynamic radius provided by the PEG [21]. This large hydrodynamic radius could create microenvironments of magnetic field inhomogeneity which would increase the effectiveness of Dy to alter the MRI signal [8, 19]. Finally, there could be a

small increase in rotational correlation time from the grafted PEG due to the large increase in molecular weight [19, 27].

Since the effect of varying the molecular weight of the PEG chain bonded to DTPA-Dy has not been studied prior to this work, the same experiments were performed using Gd for comparison. There are several instances in the literature where the molecular weight effect of PEG covalently bonded to DTPA-Gd have been studied. The general findings of these studies are that at 1.5T, DTPA-Gd has a R_2 of 5.2 $mM^{-1}s^{-1}$ and pegylated DTPA-Gd contrast agents with molecular weights of about 4.3kDa have R_2s of 6.6$mM^{-1}s^{-1}$ and 7.4$mM^{-1}s^{-1}$, which demonstrates that there is a minimal molecular weight effect on the contrast ability of DTPA-Gd [38]. In this work, T_2 studies were performed using the same synthesis pathway as contrast agents 1 and 3, substituting Gd for Dy, to repeat these literature values. At 9.4T, contrast agent 2 (5kDa) has an R_2 of 6.03$mM^{-1}s^{-1}$ and contrast agent 4 (20kDa) has a R_2 of 7.38$mM^{-1}s^{-1}$, which lie within the literature values.

These experiments were used to validate the R_2 results found for the contrast agents containing Dy (1 and 3). Using the same synthesis and experimental methods, only changing the metal ion from Dy to Gd, literature values for pegylated DTPA-Gd were repeated. This indicates that the results gathered from the Dy-containing contrast agents are due to changing the molecular weight of the PEG and not any other effect.

From the results above, it can be hypothesized that by manipulating the molecular weight of the PEG bonded to DTPA-Dy in the presence of a molecule of interest, a measurable change in T_2 would occur, leading to the detection of that molecule by MRI. In this work that molecule of interest is GSH which is an important biomolecule for maintaining the redox environment in the body. A change in the redox environment has

been implicated in several age-related pathologies and this contrast agent would allow for these diseases to be studied non-invasively.

A MRI contrast agent based on Dy was designed for the detection GSH *in vitro*. A similar design to contrast agent 3 was used with the addition of a disulfide bond between the 20kDa PEG chain and DTPA-Dy. In the presence of GSH, which contains a free thiol, the disulfide bond should undergo an exchange reaction, effectively removing the PEG chain. If the PEG chain is removed, its T_2 enhancing capabilities are no longer present and a measurable change in T_2 should occur (the R_2 of the contrast agent is expected to decrease from about 12 $mM^{-1}s^{-1}$ to about 1 $mM^{-1}s^{-1}$). The overall change in the system should be a significant increase in T_2 upon the addition of GSH.

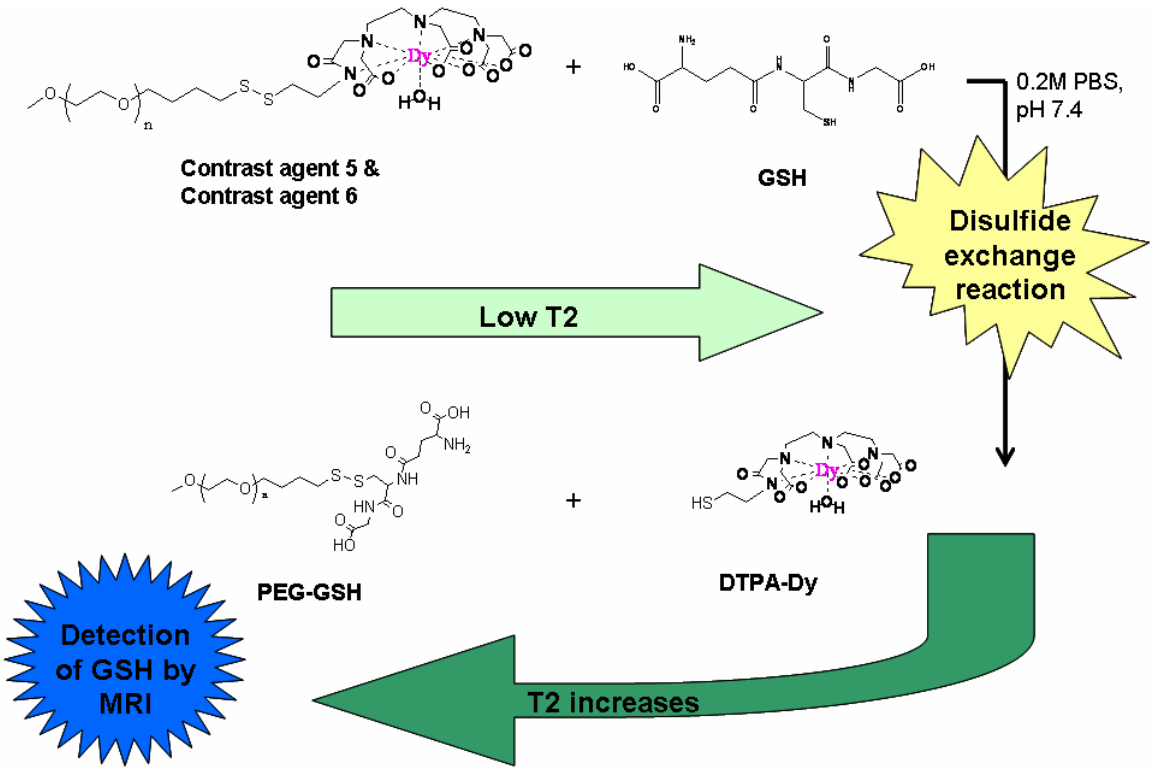

Figure 5-1 Expected route of de-pegylation of contrast agents 5 and 6 by GSH leading to a change in T_2 that can be detected by MRI

To create a GSH sensitive Dy-based MRI contrast agent, two synthesis pathways were proposed (contrast agents 5 and 6 have the same chemical structure) and are outlined in Figures 3-3 and 3-4 and discussed in the Results section. The expected mechanism for the detection of GSH with contrast agents 5 and 6 is illustrated in Figure 5-1.

Even though the steps in the synthesis of contrast agent 5 (Figure 3-3) had high yields it was ineffective in producing the desired T_2 results. The initial T_2 data collected before addition of GSH to the system had an average of 0.3189s and a standard deviation of ±18%. Five hours after the addition of GSH, the average T_2 was expected to increase towards the T_2 of pure water, which is 3s at 9.4T, instead it decreased to an average T_2

of 0.2784s ±1%. The 31.5 hour time-point did show an increase to 0.3451s ±5%, but this is not significantly different from the initial average. The data showing the decrease in T_2 after 5 hours with GSH (when it theoretically should have increased) and the large standard deviation for the initial data (±18%) indicates that the system may have been unstable at the beginning and needed some time to reach an equilibrium. This non-equilibrium system could be due to the PEG chains needing time to fully dissolve and some residual reactive free thiols that were not removed during the synthesis.

The control was designed to see if GSH could alter the T_2 of the system by itself, so a sample of contrast agent 3 (no disulfide bond) was measured before and after the addition of GSH at the same time points as the experimental samples. Over the course of the experiment, the control drifted from 0.1226s to 0.1838s, an increase of 50%. This change in T_2 may be due to a combination of a non-equilibrium initial stage, some effect of GSH on the T_2 signal, or machine error.

The second synthesis pathway (contrast agent 6, Figure 3-4) produced more promising T_2 results than contrast agent 5. High synthesis yields were also determined for this pathway.

The data followed the expected trend of increasing T_2 with time, where the average initial T_2 was 0.1889s, increasing to 0.2763s at 5 hours after the addition of GSH, and then finally increasing to 0.3230s at 24 hours post-GSH addition for a total change of 72%. The standard deviations for the initial and final time points were less than 10% indicating that the systems were in equilibrium at those time points. The 5 hour time point has a standard deviation a little above 10%, but that is acceptable because the system is still changing at this point to reach equilibrium.

The control T_2 signal also increased with time even though no GSH was added to its solution. The total increase was about 22% for the one control sample, less than the 72% change measured with samples A-C, but still large enough to be detected on an MR image.

There are several possible explanations for the ambiguity in the results of the T_2 experiments with contrast agents 5 and 6. One possible explanation is that the purification steps in the synthesis pathways may not have removed all of the free thiols from the system. Even though great care was taken during the dialysis procedures and other purification procedures, un-reacted cysteamine, for example, might have been carried along to the final product. Any residual free thiols in the system would cleave the disulfide bond in the same fashion as GSH making it indistinguishable from the reaction of the contrast agents with GSH.

The disulfide exchange reaction with GSH can produce numerous species in the reaction mixture that can compete with the desired reaction pathway (Figure 5-1). GSH can react with itself forming the oxidized version, GSSG, which decreases the number of reactive GSH molecules in the system and slows the formation of the products. Another possible undesirable side-reaction is the reaction of the DTPA-Dy product containing a free thiol with the other product, PEG-GSH, reforming the original molecule. There are numerous other side-reactions that can take place in this particular system that would either slow the reaction or compete with the desired reaction. Any of the side-reactions described here will alter the T_2 of the solution in a manner that is indistinguishable from the desired reaction producing ambiguous results. Many studies of the disulfide

exchange reaction have shown that the reaction rates for these side-reactions are often similar to the desired reaction resulting a mixture of products [42, 43, 44].

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

This work demonstrates that at a magnetic strength of 9.4T, PEG covalently bonded to DTPA-Dy increases its T_2 relaxation rate and the larger the molecular weight of the PEG chain, the higher the relaxation rate. At 9.4T, the literature value of the relaxation rate (R_2) for DTPA-Dy is approximately 1 $mM^{-1}s^{-1}$ [38], with the addition of a 5kDa PEG chain the R_2 increased to about 6 $mM^{-1}s^{-1}$ and when the molecular weight of the PEG chain was further increased to 20kDa, the R_2 of the contrast agent rose to about 12 $mM^{-1}s^{-1}$. This is an important discovery in the field of Dy-based contrast agents, because it provides a method for the R_2 of the contrast agent to be chemically manipulated.

A GSH-sensitive MRI contrast agent was designed using a 20kDa PEG chain conjugated to DTPA-Dy through a disulfide bond. It was designed so that in the presence of GSH, the PEG would be cleaved from the DTPA-Dy resulting in a decrease in R_2 that can be detected by MRI. Two synthesis pathways were used to test this design, generating contrast agents 5 and 6 (Figures 3-3 and 3-4). The synthesis pathway for contrast agent 5 proved to be ineffective and the T_2 results reflected this. 5hrs after the addition of GSH to the solutions containing 5, the T_2 of the solutions decreased, which was an unexpected result. After 31.5hrs had passed, the T_2 of the solutions increased as expected, but not enough to be significantly different from the initial data. The synthesis and T_2 results for contrast agent 6 were more promising than the results gathered from contrast agent 5. For contrast agent 6, the T_2 increased as expected for each of the time points measured for a total increase of 72%. The control for this experiment also increased over the same time points by 22%.

Future Work

To further develop upon the promising results of contrast agent 6, several steps can be taken to gather more conclusive results. In future studies, more control samples should be used to determine the significance of the experimental results over the controls. Also, more powerful purification techniques should be employed to guarantee the removal of residual reactants, especially those containing a free thiol. There may also be a possibility of redesigning the contrast agent chemically, so that after it reacts with GSH, it is no longer reactive to any free thiols in the solution.

Interestingly, in the literature, it has been found that Dy-based contrast agents *in vivo* perform more robustly than *in vitro* due to an increased compartmentalization around intact cells. The R_2 of a macromolecular Dy-based contrast agent with a molecular weight of about 5kDa is 10 to 20 times higher than the R_2s measured in this work. Future studies with GSH-sensitive MRI contrast agents should move quickly from *in vitro* studies to *in vivo* studies to take advantage of this large increase [45].

REFERENCES

[1] Brown, M. A., and Semelka, R. C., *MRI: Basic Principles and Applications.* 1999: John Wiley & Sons, New York.

[2] Weisskoff, R. M., "Physical Foundations of MR Imaging," http://airto.bmap.ucla.edu/BMCweb/CourseWork/M285/MRI/RMWFundamentals.html (Accessed December 27, 2005).

[3] Haacke, E. M., Brown, R. W., Thompson, M. R., et. al., *Magnetic Resonance Imaging: Physical Principles and Sequence Design.* 1999: John Wiley & Sons, New York.

[4] Brasch, R. C., *New Directions in the Development of MR Imaging Contrast Media.* Radiology, 1992. 183: 1 – 11.

[5] Raymond K. N., ans Pierre, V. C., *Next Generation, High Relaxivity Gadolinium MRI Agents.* Bioconjugate Chemistry, 2005. 16: 3 – 8.

[6] Hajela, S., Botta, M., Giraudo, S., et. al. *A Tris-hydroxymethyl-Substituted Derivative of Gd-TREN-Me-3,2-HOPO: An MRI Relaxation Agent with Improved Efficiency.* Journal of the American Chemical Society, 2000. 122: 11228 – 11229.

[7] Vander Elst, L., Zhang, S., Sherry, A. D., et. al., *Dy-complexes as High Field T_2 Contrast Agents: Influence of Water Exchange Rates.* Academic Radiology, 2002. 9(suppl 2): S297 – S299.

[8] Fossheim, S., Kellar, K. E., Fahlvik, A. K., et. al., *Low-molecular Lanthanide Contrast Agents: Evaluation of Susceptibility and Dipolar Effects in Red Blood Cell Suspensions.* Magnetic Resonance Imaging, 1997. 15: 193 – 202.

[9] Wang, C., Sundin, A., Ericsson, A., et. al., *Dysprosium-Enhanced MR Imaging For Tumor Tissue Characterization.* Acta Radiologica, 1997. 38: 281 – 286.

[10] Vander Elst, L., Roch, A., Gillis, P., et. al., *Dy-DTPA Derivatives as Relaxation Agents for Very High Field MRI: The Beneficial Effect of Slow Water Exchange of the Transverse Relaxivities.* Magnetic Resonance in Medicine, 2002. 47: 1121 – 1130.

[11] Toth, E., van Uffelen, I., Helm, L., et. al., *Gadolinium-based linear polymer with temperature-independent proton relaxivities: a unique interplay between the water exchange and rotational contributions.* Magnetic Resonance in Chemistry, 1998. 36: S125 – S134.

[12] Caravan, P., Greenfield, M. T., Bulte, J. W. M., *Molecular Factors That Determine Curie Spin Relaxation in Dysprosium Complexes.* Magnetic Resonance in Medicine, 2001. 46: 917 – 922.

[13] Langereis, S., de Lussanet, Q. G., van Genderen, M. H. P., et. al., *Multivalent Contrast Agents Based on Gadolinium-Diethylenetriaminepentaacetic Acid-Terminated Poly(propylene imine) Dendrimers for Magnetic Resonance Imaging.* Macromolecules, 2004. 37: 3084 – 3091.

[14] Kobayashi, H., Kawamoto, S., Jo, S-K., et. al., *Macromolecular MRI Contrast Agents with Small Dendrimers: Pharmacokinetic Differences between Sizes and Cores.* Bioconjugate Chemistry, 2003. 14: 388 – 394.

[15] Schmiedl, U., Ogan, M., Paajanen, H., et. al., *Albumin Labeled with Gd-DTPA as an Intravascular, Blood Pool-Enhancing Agent for MR Imaging: Biodistribution and Imaging Studies.* Radiology, 1987. 162: 205 – 210.

[16] Trubetskoy, V. S., Cannillo, J. A., Milshtein, A., et. al., *Controlled Delivery of Gd-Containing Liposomes to Lymph Nodes: Surface Modification May Enhance MRI Contrast Properties.* Magnetic Resonance Imaging, 1995. 13: 31 – 37.

[17] Margerum, L. D., Campion, B. K., Koo, M., et. al., *Gadolinium(III) DO3A Macrocycles and Polyethylene Glycol Coupled to Dendrimers: Effect of Molecular Weight on Physical and Biological Properties of Macromolecular Magnetic Resonance Imaging Contrast Agents.* Journal of Alloys and Compounds, 1997. 249: 185 – 190.

[18] Desser, T. S., Rubin, D. L., Muller, H. H., et. al., *Dynamics of Tumor Imaging with Gd-DTPA-Polyethylene Glycol Polymers: Dependence on Molecular Weight.* Journal of Magnetic Resonance Imaging, 1994. 4: 467 – 472.

[19] Harris, J. M., Martin, N. E., Modi, M., *Pegylation: A Novel Process for Modifying Pharmacokinetics.* Drug Delivery Systems, 2001. 40: 539 – 551.

[20] Kohler, N., Fryxell, G. E., Zhang, M., *A Bifunctional Poly(ethylene glycol) Silane Immobilized on Metallic Oxide-Based Nanoparticles for Conjugation with Cell Targeting Agents.* Journal of the American Chemical Society, 2004. 126: 7206 – 7211.

[21] Dormidontova, E. E., *Role of Competitive PEO-Water and Water-Water Hydrogen Bonding in Aqueous Solution PEO Behavior.* Macromolecules, 2002. 35: 987 – 1001.

[22] Dormidontova, E. E., *Influence of End Groups on Phase Behavior and Properties of PEO in Aqueous Solutions.* Macromolecules, 2004. 37: 7747 – 7761.

[23] Smith, G. D., Bedrov, D., *Roles of Enthalpy, Entropy, and Hydrogen Bonding in the Lower Critical Solution Temperature Behavior of Poly(ethylene oxide)/Water Solutions.* Journal of Physical Chemistry B, 2003. 107: 3095 – 3097.

[24] Yamaoka, T., Tabata, Y., Ikada, Y., *Distribution and Tissue Uptake of Poly(ethylene glycol) with Different Molecular Weights after Intravenous Administration to Mice.* Journal of Pharmaceutical Sciences, 1994. 83: 601 – 606.

[25] Yamaoka, T., Tabata, Y., Ikada, Y., *Fate of Water-Soluble Polymers Administered via Different Routes.* Journal of Pharmaceutical Sciences, 1995. 84: 349 – 354.

[26] Ladd, D. L., Hollister, R., Peng, X., et. al., *Polymeric Gadolinium Chelate Magnetic Resonance Imaging Contrast Agents: Design, Synthesis, and Properties.* Bioconjugate Chemistry, 1999. 10: 361 – 370.

[27] Doble, D. M. J., Botta, M., Wang, J., et. al., *Optimization of the Relaxivity of MRI Contrast Agents: Effect of Poly(ethylene glycol) Chains on the Water-Exchange Rates of Gd(III) Complexes.* Journal of the American Chemical Society, 2001. 123: 10758 – 10759.

[28] Lu, Z-R., Parker, D. L., Goodrich, K. C., et. al., *Extracellular Biodegradable Macromolecular Gadolinium(III) Complexes for MRI.* Magnetic Resonance in Medicine, 2004. 51: 27 – 34.

[29] Franano, F. N., Edwards, W. B., Welch, M. J., et. al., *Biodistribution and Metabolism of Targeted and Nontargeted Protein-Chelate-Gadolinium Complexes: Evidence for Gadolinium Dissociation In Vitro and In Vivo.* Magnetic Resonance Imaging, 1995. 13: 201 – 214.

[30] Mohs, A. M., Wang, X., Goodrich, K. C., et. al., *PEG-g-poly(GdDTPA-co-L-cystine): A Biodegradable Macromolecular Blood Pool Contrast Agent for MR Imaging.* Bioconjugate Chemistry, 2004. 15: 1424 – 1430.

[31] Mohs, A. M., Zong, Y., Guo, J., et. al., *PEG-g-poly(GdDTPA-co-L-cystine):Effect of PEG Chain Length on in Vivo Contrast Enhancement in MRI.* Biomacromolecules, 2005. 6: 2305 – 2311.

[32] Lu, Z-R., Wang, X., Parker, D. L., et. al., *Poly(L-glutamic acid) Gd(III)-DOTA Conjugate with a Degradable Spacer for Magnetic Resonance Imaging.* Bioconjugate Chemistry, 2003. 14: 715 – 719.

[33] Valencia, E., Hardy, G., *Practicalities of glutathione supplementation in nutritional support.* Current Opinion in Clinical Nutrition and Metabolic Care, 2002. 5: 321 – 326.

[34] Moriarty-Craige, S. E., Jones, D. P., *Extracellular Thiols and Thiol/Disulfide Redox in Metabolism.* Annual Reviews in Nutrition, 2004. 24: 481 – 509.

[35] Sechi, G., Deledda, M. G., Bua, G., et. al., *Reduced Intravenous Glutathione in the Treatment of Early Parkinson's Disease.* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 1996. 20: 1159 – 1170.

[36] Hermanson, G. T., *Bioconjugate Techniques*. 1996: Academic Press, New York.

[37] Ebright, Y. W., Chen, Y., Kim, Y., et. al., *S-[2-(4-Azidosalicylamido)ethylthio]-2-thiopyridine: Radioiodinatable, Cleavable, Photoactivatible Cross-Linking Agent.* Bioconjugate Chemistry, 1996. 7: 380 – 384.

[38] Unger, E. C., Shen, D., Wu, G., et. al., *Gadolinium-containing Copolymeric Chelates – A New Potential MR Contrast Agent..* MAGMA, 1999. 8:154 – 162.

[39] Aime, S., Botta, M., Fasano, M., et. al., *Prototropic and Water-Exchange Processes in Aqueous Solutions of Gd(III) Chelates.* Accounts of Chemical Research, 1999. 32: 941 – 949.

[40] Aime, S., Botta, M., Fasano, M., et. al., *Lanthanide(III) chelates for NMR biomedical applications.* Chemical Society Reviews, 1998. 27: 19 – 29.

[41] Meade, T. J., Taylor, A. K., Bull, S. R., *New magnetic resonance contrast agents as biochemical reporters.* Current Opinion in Neurobiology, 2003. 13: 597 – 602.

[42] Whitesides, G. M., Lilburn, J. E., Szajewski, R. P., *Rates of Thiol-Disulfide Interchange Reactions Between Mono- and Dithiols and Ellman's Reagent..* Journal of Organic Chemistry, 1977. 42: 332 – 338.

[43] Szajewski, R. P., Whitesides, G. M., *Rate Constants and Equilibrium Constants for Thiol-Disulfide Interchange Reactions Involving Oxidized Glutathione..* Journal of the American Chemical Society, 1980. 102: 2011 – 2026.

[44] Rabenstein, D. L., Weaver, K. H., *Kinetics and Equilibria of the Thiol/Disulfide Exchange Reactions of Somatostatin with Glutathione..* Journal of Organic Chemistry, 1996. 61: 7391 – 7397.

[45] Zaharchuk, G., Bogdanov Jr., A. A., Marota, J. J. A., et. al., *Continuous Assessment of Perfusion by Tagging Including Volume and Water Extraction (CAPTIVE): A Steady-State Contrast Agent Technique for Measuring Blood Flow, Relative Blood Volume Fraction, and the Water Extraction Fraction.* Magnetic Resonance in Medicine, 1998. 40: 666 – 678.